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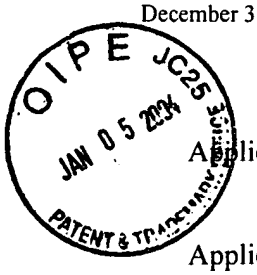
December 31, 2003

PATENT APPLICATION
Attorney's Docket No.: 3033.1002-001



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Darrell H. Carney, Roger S. Crowther, David J. Simmons, Jinping Yang and William R. Redin		
Application No.:	09/909,122	Group Art Unit:	1647
Filed:	July 19, 2001	Examiner:	DeBerry, R.M.
Confirmation No.:	1024		
For:	STIMULATION OF BONE GROWTH WITH THROMBIN PEPTIDE DERIVATIVES		

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, or is being facsimile transmitted to the United States Patent and Trademark Office on:

12-31-03 Dawn M Myers
Date Signature

Dawn M. Myers
Typed or printed name of person signing certificate

TRANSMITTAL OF DECLARATION OF DARRELL H. CARNEY, PH.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Please find herewith an executed Declaration of Darrell H. Carney, Ph.D. under 37 C.F.R. §1.132 with Figure 1 and Exhibits A to C for filing in the above referenced patent application.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By _____
Helen Lee
Registration No. 39,270
Telephone (978) 341-0036
Facsimile (978) 341-0136

Concord, Massachusetts 01742-9133
Dated: December 31, 2003

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Darrell H. Carney, Roger S. Crowther, David J. Simmons, Jinping Yang and William Redin		
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12-31-03 Dawn M Myers
Date Signature

Dawn M Myers
Typed or printed name of person signing certificate

DECLARATION OF DARRELL H. CARNEY, PH.D.
UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Darrell H. Carney, Ph.D., of 1125 Tallow Drive, Dickinson, Texas 77539, U.S.A., declare and state that:

1. I am one of the inventors of the subject matter described and claimed in U.S. Application No. 09/909,122 ('122), filed July 19, 2001.

2. I have been on the faculty at the University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555, U.S.A. since 1978, most recently as a Professor and Vice Chairman in the Department of Human Biological Chemistry and Genetics. I am also founder, President and Chief Executive Officer of Chrysalis BioTechnology Inc., 220 Market Street, Suite 605, Galveston, Texas 77550, U.S.A. A copy of my curriculum vitae, which describes my educational and professional experience, is attached as Exhibit A.

I have published extensively in refereed publications, most of which have focused on the role of thrombin, thrombin peptides and thrombin receptors in cellular regulation. A list of publications authored or co-authored by me is included as part of my curriculum vitae.

3. I have found that ***osteoblasts have high-affinity thrombin receptors and respond to compounds such as TP508 which activate the non-proteolytic thrombin cell surface receptor (NPAR)*** but do not have proteolytic activity to activate the proteolytically activated receptors (PAR1-PAR4). I have also found that ***compounds that activate NPAR stimulate osteoblast proliferation***.

The following is a description and discussion of the experimentation performed by me or under my supervision and of the results which demonstrate that osteoblasts have high-affinity thrombin receptors and respond to NPAR agonists resulting in the stimulation of osteoblast proliferation.

Thrombin Binding to Mouse Osteoblasts

The specific binding of ^{125}I thrombin to MC3T3-E1 (subclone 4) osteoblasts (ATCC No. CRL-2593) was carried out using established thrombin receptor binding assays as disclosed in U.S. Patent No. 5,352,664 and Carney, D.H. and Cunningham, D.D., *Cell*, 15:1341-1349 (1978). Briefly, highly purified thrombin was iodinated and added to cultures of osteoblasts with or without unlabeled thrombin to correct for nonspecific binding. By incubating cells with different concentrations of labeled thrombin and measuring the amount

of thrombin bound to cells and the amount of free thrombin in the medium, it is possible to estimate the number of receptors per cell and the affinity of thrombin for that binding site.

Scatchard analysis of labeled thrombin binding from two separate experiments indicate that mouse MC-3T3 osteoblasts have two classes of high-affinity receptors on their cell surfaces with an average of 83,000 very high affinity binding sites per cell (Kd = ~230 pM) and 180,000 high affinity sites (Kd = ~16.8 nM). This binding is similar to that reported for high-affinity thrombin binding to fibroblasts (Carney, D.H. and Cunningham, D.D., *Cell*, 15:1341-1349 (1978)), and for which TP508 competes for binding (U.S. Patent No. 5,352,664 and Glenn, K.C. *et al.*, *J. Peptide Research*, 1:65-73 (1989)) to initiate proliferative signals.

NPAR Agonist Stimulation of Mouse Osteoblast Proliferation

To determine the effect of NPAR agonists on osteoblast proliferation, MC3T3-E1 (subclone 4) osteoblasts (ATCC No. CRL-2593) were seeded into 24 well plates at a density of 5.0×10^4 cells per well and were cultured in DMEM with 10% fetal calf serum. After 24 hours, the medium was removed and non-adherent cells were removed by rinsing the cultures gently with 1 ml of serum free DMEM (0% serum) and replacing the medium with 1 ml of DMEM. After 48 hours in serum free medium, the NPAR agonist TP508 (SEQ ID NO: 5) was added at the indicated concentrations from 0 to 100 μg per ml (Tables 1 and 2) and cell number was determined 48 hours later using a Coulter Cell Counter as described in Carney, D.H. and Cunningham, D.D., *Cell*, 15:1341-1349 (1978). As shown in Tables 1 and 2, addition of TP508 increased cell number in two separate experiments relative to that seen in untreated control osteoblasts (0 μg) by approximately 20%. The results from the two experiments are also shown in Figure 1.

Table 1: Experiment 1

Treatment	Cell Number (cells/well) ± SD
TP508 (0 μg)	39696.7 ± 3266.1
TP508 (10 μg)	44500.0 ± 4217.1
TP508 (30 μg)	46420.0 ± 2271.1
TP508 (100 μg)	48306.7 ± 1804.9

Table 2: Experiment 2

Treatment	Cell Number (cells/well) ± SD
TP508 (0 μg)	43070.0 ± 2358.5
TP508 (10 μg)	44906.7 ± 1026.9
TP508 (30 μg)	46493.3 ± 3890.5
TP508 (100 μg)	47380.0 ± 698.7

4. The subject application (the '122 application) discloses results which demonstrate that compounds which activate NPAR are osteoinductive. The application discloses results which demonstrate that NPAR agonists can stimulate bone growth at a site within a subject at which bone growth would not occur if the site were left untreated. In particular, the application discloses results which demonstrate that TP508 is osteoinductive and induces bone formation in sites where bone formation did not occur without treatment (see, e.g., Examples 1 and 2).

5. U.S. Patent No. 5,352,664 ('664) and U.S. Patent No. 5,500,412 ('412) (copies of which are attached hereto as Exhibits B and C), of which I am an inventor of the subject matter described and claimed therein, set forth experiments and results which demonstrated that epithelial cells and fibroblast cells have high-affinity thrombin receptors and that thrombin peptide derivatives (NPAR agonists), including TP508, can bind specifically to the thrombin receptors (see Example 4). These earlier applications also disclose results which

demonstrated that NPAR agonists, including TP508, acting on the thrombin receptors, can induce DNA synthesis and promote proliferation of these cells (see Example 5).

6. The '664 and '412 patents demonstrated that: (1) NPAR receptors are present on epithelial cells and fibroblast cells; and (2) activation of NPAR receptors by agonists, such as TP508, stimulate proliferation of these cells.

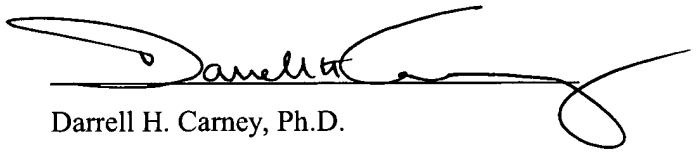
The results described herein in paragraph 3 provide evidence showing that: (1) NPAR receptors are present on osteoblasts; and (2) TP508 stimulates osteoblasts to proliferate. The subject ('122) application provides evidence showing that TP508 is osteoinductive, stimulating bone formation in sites where bone formation did not occur without treatment.

From the data, I conclude that activation of NPAR causes osteoblasts to proliferate. Therefore, agonists other than TP508, e.g., those which act on epithelial and fibroblast cells, would also cause osteoblast proliferation. I conclude that NPAR agonists other than TP508 would be osteoinductive and stimulate bone formation in sites where bone formation would not occur without treatment. I further conclude that analogs of TP508, such as those recited in claims of the '664 and '412 patents, would stimulate osteoblast proliferation. I also conclude that analogs of TP508 such as those recited in claims of the '664 and '412 patents are expected to be osteoinductive, stimulating bone formation at sites where osteoinduction is needed (i.e., at sites where bone formation would not occur if the sites were left untreated).

7. Since analogs of TP508 have been shown to activate NPAR, analogs of TP508 would also be expected to stimulate osteoblasts, given the evidence that NPAR receptors are present on osteoblasts and TP508 stimulates osteoblasts to proliferate. Thus, I conclude that analogs of TP508 would stimulate osteoblast proliferation, and be osteoinductive, stimulating bone formation at sites where osteoinduction is needed (i.e., at sites where bone formation would not occur if the sites were left untreated).

8. I declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Moreover, these statements are made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Darrell H. Carney, Ph.D.

December 30, 2003

Date

Attachments

Exhibit A	Curriculum vitae, including list of publications
Exhibit B	U.S. Patent No. 5,352,664 ('664)
Exhibit C	U.S. Patent No. 5,500,412 ('412)

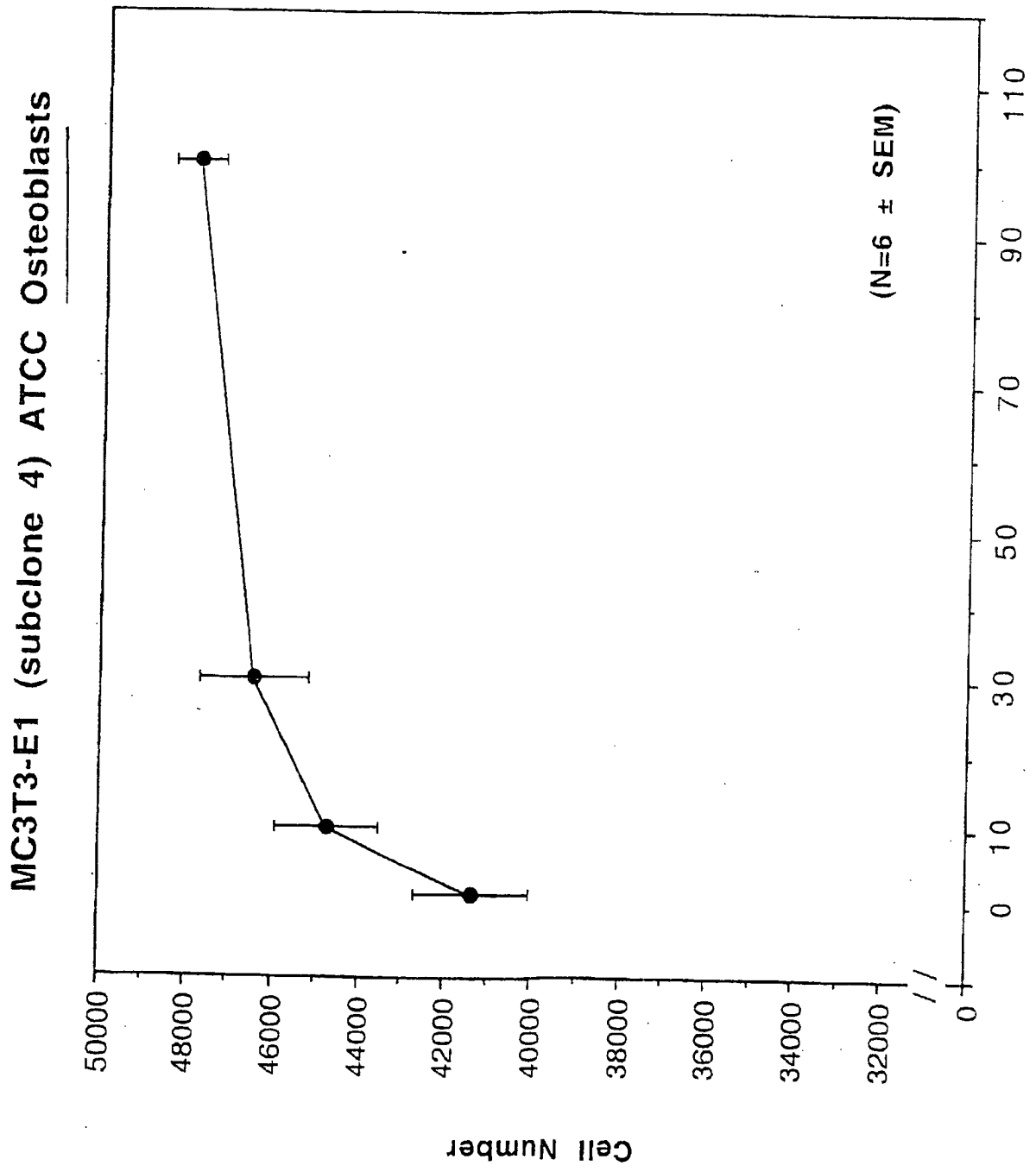


FIG. 1

CURRICULUM VITAE

NAME: **Darrell Howard Carney** DATE: January 1, 2003

PRESENT POSITION AND ADDRESS:

Professor and Vice Chairman (September 2000)
Department of Human Biological Chemistry and Genetics
The University of Texas Medical Branch
Galveston, TX 77555-0645
(August, 1978)
Phone: (409) 772-3210
Fax: (409) 772-2348
Email: dcarney@utmb.edu

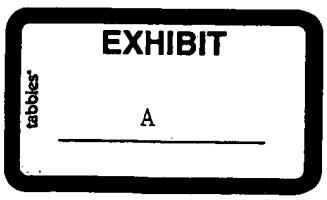
Chrysalis BioTechnology, Inc. (November 1995)
2200 Market, Suite 600
Galveston, TX 77550
Phone: (409) 750-9251
Fax: (409) 750-9253
Email: dcarney@chrysalisbio.com

BIOGRAPHICAL:

Date and Place of Birth:	April 15, 1948, Boise, Idaho
Citizenship:	USA
Social Security Number:	518-52-7622
Home Address:	1125 Tallow Drive Dickinson, Texas 77539
Phone:	(281) 534-3276
Fax:	(281) 337-4832

EDUCATION:

Institution	Date	Major	Degree
Northwestern University Evanston, Illinois	1966-68	Biology	
College of Idaho Caldwell, Idaho	1968-70	Biology	B.S.
University of Connecticut Storrs, Connecticut	1970-75	Developmental Biology	Ph.D.
University of California Irvine, California	1975-78	Cell Biology	Postdoctoral



PROFESSIONAL AND TEACHING EXPERIENCE:

1971-1972	Teaching Assistant in Developmental Biology and Human Anatomy, University of Connecticut
1972-1975	NIH Predoctoral Trainee Cell Biology Training Grant - GM 00317
1975-1978	NIH Postdoctoral Fellowship University of California, Irvine - CA 12306
1976-1978	Instructor in Medical Microbiology University of California, Irvine
1978-1982	Assistant Professor, Biochemistry Division, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX (August 1978)
1978-Pres.	Biochemistry Program, Graduate School of Biomedical Sciences The University of Texas Medical Branch, Galveston, TX (August 1978)
1982-1992	Associate Professor, Division of Biochemistry The University of Texas Medical Branch, Galveston, TX (September 1982)
1986-1998.	Director of UTMB Peptide-DNA Synthesis Laboratory The University of Texas Medical Branch, Galveston, TX
1987-1988	Co-Director, UTMB Cancer Center Program; Hormone-Receptor Interactions in Cancer. The University of Texas Medical Branch, Galveston, TX
1992-Pres	Professor, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX (September 1992)
1994-1995	Founder, Gal Tech Wound Therapies, DBA. 201 University Blvd. Suite 924, Galveston, TX (July, 1994)
1995-Pres	Founder and Scientific Director, Chrysalis BioTechnology, Inc. 2200 Market, Suite 600, Galveston, TX 77550 (November, 1995)
1997-Pres	President and CEO, Chrysalis BioTechnology, Inc. 2200 Market, Suite 600, Galveston, TX 77550 (July, 1997)
1998-Pres	Partner, Emprise Scientific, DBA of Emprise Partners, LTD. 1125 Tallow Drive, Dickinson, TX 77539 (July 1998)
2000-Pres	Vice Chairman, Department of Human Biological Chemistry & Genetics, UTMB. (September, 2000)

RESEARCH ACTIVITIES:

1968-1970 Undergraduate, College of Idaho, Biology Department
Independent Research, Funded by the Idaho Heart Association.

1970-1975 Graduate Research, University of Connecticut, Storrs, Connecticut. Departments of Animal Genetics and Genetics and Cell Biology, Norman W. Klein, Advisor. Biochemistry and Developmental Biology of brain development.

1975-1978 Postdoctoral Research, The University of California, Irvine, Department of Medical Microbiology,
Dennis D. Cunningham, Advisor Regulation of cell proliferation. Studies led to discovery and identification of thrombin receptors on the surface of fibroblasts and other cells.

1978-Pres. The University of Texas Medical Branch, Department of Human Biological Chemistry and Genetics. Role of thrombin receptors and thrombin-derived peptides in regulating cellular activities as they relate to inflammation, tissue repair, and cancer.

Based on our initial discovery of thrombin receptors on cells, our laboratories have studied the activation of these receptors and the subsequent signal cascades initiated by proteolytic and non-proteolytic thrombin interactions with cells. These studies have demonstrated that thrombin interacts with and activates a non-proteolytically activated thrombin receptor (NPAR) that is distinct from the proteolytically activated receptors (PAR1-4). Using synthetic peptides we identified the high-affinity binding domain of thrombin and discovered that the thrombin peptide TP508, representing this domain, activates NPAR and stimulates specific cellular activities that accelerate tissue repair. This peptide, also known as Chrysalin®, has been tested in Phase II safety and efficacy human clinical trials for accelerating the healing of chronic diabetic ulcers and orthopedic (distal radius) fractures. Based on positive results from these first trials, Phase II (diabetic ulcer) and Phase III (fresh fracture) trials will be initiated in 2002 by Chrysalis BioTechnology and its strategic partners Abbott Laboratories and OrthoLogic. In addition, human clinical trials to test the efficacy of thrombin peptides in spine fusion, cartilage repair, and myocardial revascularization are planned for initiation in 2002.

Because TP508 is proving to be an effective and potentially important molecule for orthobiologics, dermal tissue repair, prevention of vascular restenosis and revascularization of ischemic heart, basic science studies in our laboratory and in the laboratories of our collaborators are focusing on: (i) understanding the signal transduction pathways stimulated by activation of the non-proteolytically activated thrombin receptor (NPAR) in different tissues using array analysis and other techniques; (ii) cloning the NPAR receptor; (iii) and developing validated cell assays to screen peptide analogues and mimetics for activity.

RESEARCH SUPPORT

A. Previous Support

1978-79	Institutional Biomedical Research Support Grant DHEW 5-S07RR05427	$ 7,200
1978-79	Cancer Center Core Grant (CA 17701-04) "Thrombin Receptors in Normal and Transformed Cells"	8,750
1978-79	American Cancer Society Institutional Research Grant No. IN 112B	2,950
1979-80	UTMB Cancer Center - "Video Intensification of Cell Surface Molecules"	17,000
1979-82	DHEW 1R01-AM-25807, (01-03) "Role of Cell Surface in Regulating Cell Proliferation."	164,307
1982-87	DHHS 1 K04 CA00805, (01-05) Research Career Development Award	190,050
1982-87	DHHS 2R01 AM 25807, (04-08) "Role of Cell Surface in Regulating Cell Proliferation"	380,869
1983	Intramural Grant "Microinjection of Macromolecules into Single Living Cells."	21,500
1984-85	NSF PMC-8400954 "Acquisition of a Gas-Phase Protein Sequencer" (Co-P.I.)	65,000
1984-88	DHHS 1R01 GM 33505 "Studies of Cytoplasmic Microtubule Heterogeneity" (Co-investigator, 5% effort)	228,662
1985-86	Texas Neurofibromatosis Foundation, "Autocrine Stimulation of Neurofibromatosis by Growth Factors or Their Receptors."	9,091
1986-88	UTMB Administrative Support Grant, "Peptide and Oligonucleotide Synthesis Laboratory"	180,000
1987-88	DRR-BRS 1-S10RR03469, Principle Investigator "UTMB Peptide Synthesizer Facility"	95,500
1987-1997	UTMB Administrative Yearly Support Grant, "Peptide and Oligonucleotide Synthesis Laboratory"	30,000
1988-89	Monsanto Co./Searle, "Thrombin Peptides as Biological Response Modifiers"	40,000

1988-90	Texas Advanced Technology Program, "Thrombin and Synthetic Peptides in Wound Healing and Modulation of Biological Responses"	178,490
1989-92	Texas Advanced Technology Program, P. I., "Thrombin Peptides as Enhancers of Tissue and Bone Healing, and Inhibitors of Adhesions and Vascular Permeability"	200,000
1989-92	J. D. Searle. "Preclinical Evaluation of Thrombin Peptides as Enhancers of Wound Healing"	60,000
1987-93	DHHS 2R01 DK 25807 (09-15), "Role of Cell Surface in Regulation of Proliferation"	736,227
1992-94	American Diabetes Association, "Possible Acceleration of Diabetic Wound Healing with Thrombin and Synthetic Thrombin Receptor Activating Peptides"	79,941
1992-95	Johnson & Johnson Medical Inc., "Evaluation of Immobilized-TRAP-508 as a Wound Healing Device	43,976
1993-98	RO1-GM47572 "Role of Thrombin Peptides in Regulating Wound Healing." (P.I.) 5-years	591,950
1995	1 R43 AI38153-01 "Thrombin Peptide Effect on Cellular Antimicrobial Action" (Co Investigator, J. Stiernberg, Ph.D., P. I.)	100,000
1997-01	NIH 1R44-AI-38153 SBIR Phase II grant "Antimicrobial action of TRAP-508 (DHC, Co-Investigator, Janet Stiernberg, Ph.D., PI)	$750,000
1999-01	NIH-SBIR Phase I grant "Accelerated Fracture Repair Using Thrombin Peptides" (DHC-Scientific Director, Roger Crowther, PI)	$100,000
1997-01	NIH 1R44-DK 53580 SBIR Phase I/Phase II " Effect of Thrombin Peptides on Chronic Wounds" (DHC-Scientific Director, Laurie Sower, Ph.D., PI)	$850,000
1999-01	1 R 43 HL64508-01 NIH-SBIR Phase I Grant (A Norfleet, PI) "Inhibition of vascular restenosis by the TP508 peptide" (DHC-Scientific Director, Co-investigator)	$100,000
1999-01	1 R 43 AR46343-01 NIH-SBIR Phase I Grant (J. Stiernberg, PI) Cartilage repair induced by thrombin peptide TP508 (DHC-Scientific Director, Co-investigator)	$100,000

B. Current Support

D. H. Carney, Principal Investigator

1999-2003 CHR-001 "Molecular Mechanisms of Thrombin in Wound Healing, Inflammation, and Vascular Repair" Chrysalis BioTechnology, Inc. (P.I.) 800,000

D. H. Carney, Co-PI/Co-Investigator

1999-02 1R 44 AR 45508-02 NIH-SBIR Phase II Grant $750,000
"Accelerated Bone Repair by a Synthetic Thrombin-Derived Peptide" (DHC-Scientific Director, Roger Crowther, PI)

2001-02 1R 43 HL69661-01 NIH-SBIR Phase I Grant $100,000
"Revascularization of Ischemic Heart Tissue by TP508"
(DHC- Co-Investigatior, Chris Coleman, PI)

C. Pending Support

1 R 44 HL64508-02 NIH-SBIR Phase II Grant (C. Coleman, PI)
"Inhibition of vascular restenosis by the TP508 peptide"
(DHC-Scientific Director, Co-investigator) $750,000

1 R 44 NIH SBIR Phase I Grant (M. Keherly, PI) entitled "Enhanced Antimicrobial Activity by Synthetic Peptide NTP"
(DHC, Co-investigator) $100,000

D. Patent Applications/Inventions

1986 "Thrombin Polypeptides :Composition and Methods for Use", **Darrell H. Carney** and Kevin C. Glenn, US. Patent Issued (5, 925,201) October 4, 1994. Issued, 10/04/94 Patent No 5,352,664.

1987 "Thrombin Peptides which Modulate Receptor Occupany and Mitogenic Stimulation", **Darrell H. Carney** and Kevin C. Glenn.
European Patents 87 907 652.9-2110 (US87/02882), Issued.

1986 "Use of a Radiolabeled Monoclonal or Monovalent F(ab) Fragments of Monoclonal Antibodies for Quantitation of Cytoskeletal Antigens" (Invention Disclosure), WC Thompson, **DH Carney** and RL Ball.

1994 "Thrombin Peptides which Modulate Receptor Occupancy and Mitogenic Stimulation", Divisional Application for Use in Wound Healing. **Darrell H. Carney** and Kevin C. Glenn (#UTSG-043),
Div. of (5, 925,201). US. Patent Issued Number 5, 500,412, March 19,1996.

1994 "Synthetic Peptide Neutrophil Cell Chemotactic Agents" **Darrell H. Carney** and Shyam Ramakrishnan (Disclosed to UTMB August, 1994), Patent Application 08/330,594 filed October 28,1994 (DC-006) by Chrysalis BioTechnology, Issued 10/30/01.

1995 "Thrombin Polypeptides: Composition and Methods for Use", **Darrell H. Carney** and Kevin C. Glenn, Divisional application for anti metastatic and inhibitory use of thrombin peptides to prevent unwanted proliferation or alteration of cellular function. (Pending).

2000 "Thrombin Derived Polypeptides: Compositions and Methods for Use. **Carney, D.H.** and Glenn, KC. Divisional Application #3033.1001-003 filed 8/02/00

2000 "Thrombin Derived Polypeptides: Compositions and Methods for Use. **Carney, D.H.** and Glenn, KC. Divisional Application #3033.1001-004 filed 8/02/00

2000 "Method of therapy with Thrombin Derived Peptides" **Carney, D.H.** Provisional Application for use of thrombin peptides in cardiovascular repair, inhibition of restenosis and myocardial revascularization. #3033.1000-000 Filed 07/12/00.

2000 "Stimulation of Bone Growth with thrombin peptide derivatives" **Carney, DH.** , Crowther, R., Simons, D., Redin, WR., Yang,J. Provisional application for use of thrombin peptides in repair of bone segmental gap filling, spinal fusion and areas where new bone growth are required. #3033.1002-000 Filed 7/19/00.

2000 "Stimulation of Cartilage Growth with agonists of the non-proteolytically activated thrombin receptor. **Carney, D.H.,** Crowther, R., Stiernberg, J., and Bergmann, J. Provisional application for use of thrombin peptides in cartilage and ligament repair, disc repair, etc. # 3033.1003-000 (60/219.800) filed 7/20/00.

2001 "Synthetic Peptide Neutrophil Cell Chemotactic Agents" **Darrell H. Carney** and Shyam Ramakrishnan (Continuation in part) filed June 2001

2001 "Method of therapy with Thrombin Derived Peptides" **Carney, D.H.** US, European PCT, Tiawan, and Thialand Applications for use of thrombin peptides in cardiovascular repair, inhibition of restenosis, and myocardial revascularization. #3033.1000-000. Filed on 07/12/01.

2001 "Stimulation of Bone Growth with thrombin peptide derivatives" **Carney, DH.**, Crowther, R., Simons, D., Redin, WR., Yang,J. US and European PCT application s for use of thrombin peptides in repair of bone segmental gap filling, spinal fusion and areas where new bone growth is required. #3033.1002-000. Filed on 7/19/01.

2001 "Stimulation of Cartilage Growth with agonists of the non-proteolytically activated thrombin receptor. **Carney, D.H.**, Crowther, R., Stiernberg, J., and Bergmann, J. US and European PCT for use of thrombin peptides in

cartilage and ligament repair, disc repair, etc. # 3033.1003-000 (60/219.800) filed 7/20/01.

2001 "Method for promoting healing of diabetic ulcers." **Carney, D.H.,** Provisional US Application based on results of human diabetic ulcer trials. #3033.1008-000. Filed on 7/27/2001.

COMMITTEE RESPONSIBILITIES

A. National Committees/Editorial Advisory Boards/Manuscript Reviews, Etc.

1978-Pres.	Ad Hoc Reviewer of Manuscripts for: J. Biol. Chem., J. Cell. Biochem., J. Cell Biology, J. Cell. Physiol., J. Clin. Invest., FASEB Journal, Cancer Research, Lab. Investigation, Molecular Endocrinology, Nature, Federation Proceedings, Biochem. J., J. Pharmacological Res., Cell Motility and Cytoskelton, and National Science Foundation Grants.
1982	National Institute of Allergy and Infectious Diseases, Transplantation Biology and Immunology, Subcommittee (Program Project Study Section) (Ad Hoc Member)
1986	Neurological Sciences 1 Ad Hoc-2 Study Section
1986-90	Editorial Advisory Board, Molecular Endocrinology
1989	National Heart, Lung and Blood Institute, Program Project Site Visit (Albany, NY).
1989	Oklahoma Center for the Advancement of Science and Technology, Member, Study Section, (March, 19-21).
1989	Oklahoma Center for the Advancement of Science and Technology, Chair, Biomedicine/Biotechnology Study Section, (October 15-17).
1989-91	Consultant, J.D. Searle and Co., Wound Healing Agents.
1990	Oklahoma Center for the Advancement of Science and Technology, Chair of Chairs, Biomedicine/Biotechnology Study Session, (Feb. 18-20).
1991	Oklahoma Center for the Advancement of Science and Technology, Biomedicine - Biotechnology Study Session, (February)
1991-1997.	Consultant, Oklahoma Center for Advancement of Science and Technology
1992	NIH Clinical Sciences Study Section, subcommittee.
1994	NIH GM Special Study Section, Chronic Wound Healing.
1994-1995	Founder and Scientific Director, Gal Tech Wound Therapies.
1995-Pres.	Founder and Scientific Director, Chrysalis BioTechnology, Inc.
1998-99.	Wound Healing Society Program Committee

B. UTMB Committees

1. Graduate School of Biomedical Sciences Committees

1980-1988 Graduate Program Review Committee
1981 Vice-Chairman
1982-1988 Chairman
1988-1996 Scholarship Committee.
1992-1996 Chairman
1992-1996 Graduate Recruitment Committee

Advancement to Candidacy, Examination Committees

1979	Randall Kohl	Biochemistry
1980	John Scott Somerset	Genetics & Cell Biology
1980	Helena Hwu	Biochemistry
1980	Kathryn L. Crossin	Biochemistry
1981	Craig S. Woodard	Genetics & Cell Biology
1982	Gregory R. Alsip	Genetics & Cell Biology
1983	Rampyari Raja	Biochemistry
1983	Robin Cooper	Genetics & Cell Biology
1984	Gloria Frost	Biochemistry
1985	Hillary Heard	Microbiology
1985	Eve Johnson	Microbiology
1985	Jonathan Lloyd	Anatomy
1986	Eric Gordon	Biochemistry
1986	Gulzar Sandhu	Biochemistry
1986	Jonathan Lloyd	Anatomy
1987	Jerome Choate	Neuroscience
1989	Olapade James	Biochemistry
1990	Shyam Ramakrishnan	Biochemistry
1990	David Scott	Genetics & Cell Biology
1992	Dennis Kim	Biochemistry, Genetics & Cell Biology
1992	David Millinoff	Microbiology
1994	Laurie Sower	Microbiology
1996	David Hester	HBC & G
1997	Christie Bogolin	HBC & G

Masters Degree Supervisory Committees

1981-1982 M. Sheila Trumble, Pathology
1981-1983 Rebecca Ball, Microbiology
1988-1988 Nora Davis, Biochemistry, Supervisor
1989-1990 Fang Wang, Genetics & Cell Biology, Supervisor
1992-1992 Vanessa Paulley, Biochemistry, Genetics & Cell Biology, Supervisor

Ph.D. Supervisory Committees

1979-1980 John M. Nickerson, Genetics & Cell Biology
1980-1982 Kathryn L. Crossin, Biochemistry, Supervisory Professor,
1982-1984 Janet Stiernberg, Biochemistry, Research Supervisor
1982-1986 Robin Cooper, Cell Biology
1982-1986 Gregory R. Alsip, Genetics & Cell Biology
1984-1986 Rampyari Raja, Biochemistry

1982-1987 Hillary Heard, Microbiology
1983-1987 Rebecca Ball, Microbiology, Research Supervisor
1984-1987 Debra Morris, Preventive Medicine and Community Health, Research Supervisor
1985-1987 Sang-Uk Nham, Human Genetics & Cell Biology
1985-1991 Stephen Pearson, Biochemistry
1985-1988 Lawrence Smith, Microbiology
1986-1987 Gloria Herbosa, Biochemistry, Supervisory Professor
1986-1989 Eve Johnson, Microbiology
1986-1989 Eric Gordon, Biochemistry, Supervisory Professor
1987-1987 Johnathan Lloyd, Anatomy
1987-1990 Jerome Choate, Neuroscience
1988-1991 Alexandra Kemendy, Physiol & Biophys
1990-1995 David L. Scott, Human Genetics & Cell Biology, M.D./Ph.D. Program. S. Professor
1991-1994 Olapade James, Biochemistry, Genetics & Cell Biology, Supervisory Professor.
1992-1994 Shyam Ramakrishnan, Biochem Genetics & Cell Biology, Supervisory Professor.
1992-1994 Dennis Kim, Biochemistry, Genetics & Cell Biology, M.D./Ph.D. Program. Supervisory Professor.
1994-1995 Laurie Sower, Microbiology.
1994-1995 Juan Yu, Neurobiology.
1997- 1999 BoJoy Yohanna, Microbiology
1997-1999 Kevin Bobbitt, Microbiology

2. School of Medicine Committees
a. Past Committee Service

1981 Search Committee to select Chairman of Radiation - Cancer Therapy Department
1981 Search-Advisory Committee to select Director of Academic Computing and Biostatistics
1982-1983 Academic External Review Committee to review the Department of Anatomy
1983 Academic External Review Committee to review the Department of Microbiology
1984-1985 Faculty Advisory Committee, National Student Research Forum
1986 Search Committee to select Dean of the Graduate School and Research Vice-President
1987 External Review Panel to review the Department of Pharmacology
1987-1990 Elected Member of the Academic Planning Committee
1991 LCME Subcommittee for Self Study and Accreditation
1991-1993 Elected Member, Faculty Coordinating Council
1991-1993 Chair, Faculty Coordinating Council
1991-1993 Voting Member, Executive Committee of the Faculty of Medicine
1992-1993 Member Search Committee, Vice President for Public Relations and External Affairs
1984-1993 Intellectual Properties Committee (Patent Review Committee)
1986-1993 Chairman, Intellectual Properties Committee
1990-1993 Faculty Advisory Council -- Dean of Medicine
1989-1996 Advisory Committee for Continuing Medical Education
1995-1996 Nominating Committee

1996-1999. Technology Advisory Committee
1997-1999 Curriculum Committee Task Force - Dermal/Wound healing

b. Current Committee Responsibilities

3. Departmental Committees
a. Past Departmental Committee Service

1979 - 1985 Admission and Graduate Recruitment Committee - Biochemistry
1979 - 1990 Biochemistry Curriculum Committee
1982 - 1983 Departmental Travel Committee
1984 - 1990 Chairman, Biochemistry Credentials Committee
1986 - 1987 Departmental Recruitment Committee
1986 - 1988 Chairman's Advisory Committee
1989 - 1990 Departmental Recruitment Committee
1990 - 1992 HBC&G Graduate Program Credentials Committee
1991 - 1993 HBC&G Departmental Travel Committee
1991 - 1993 HBC&G Departmental Faculty Recruitment Committee
1993 - 1994 HBC&G Space Advisory Committee
1993 - 1995 Graduate Program Credentials Committee
1994 - 1997 Graduate Program Examination Committee
1995 - 1997 Chair, Graduate Program Exam. Committee
1995 - 1996 Chairman's Advisory Committee
1997-2000 Graduate Program Curriculum Committee

b. Current Departmental Committee Responsibilities

1998-Pres Compensation Advisory Committee
1999-Pres Chairman's Advisory Committee
1999-Pres Vice Chairman, Dept. of HBC&G
2000-Pres Department APT Committee

TEACHING RESPONSIBILITIES AT UTMB

A. Medical School

1987-1998 Medical Biochemistry, Cells and Genes 6501 - Lecture and SGSS on Cell Surface Receptors, Transport and Transmembrane Signals (five Lectures)

B. Graduate School

1979-1996 Biochemistry 6602 - Graduate Biochemistry Regulation and Control of Intermediary Metabolism (eight Lectures)

1979-1992 Biochemistry 6306 - Advanced Biochemistry Laboratory, Course Coordinator

1984-99 Fundamentals of Cell Biology 6407 - Receptor-Cytoskeletal Interaction, Transmembrane Signaling (4 lectures)

1991-97	HBC&G Special Topics, Growth Factors and Interleukins in Cellular Regulation. Course Co-coordinator (~20 hr of lecture, Course taught 1991, 92, 93, 95, 97).
1993- 1998	Cell Bio Program - Cell biology - Growth Factors and Cell Cycle Regulation (two lectures)
1993- 1996	Cell Bio Program- Biochemistry - "Energy and Intermediary Metabolism" and "Glycolysis" (two lectures)
1999 - present	BBSC Cell Biology 6204 Cell Cycle Regulation 4- lectures and/or one small group (alternating years).
2000-present	BBSC 6116 Inflammation Module, course co-director
2000-present	Cell Signaling Course, Co-director (~18 hours)

C. Current Graduate-Medical Students in Lab Training/Projects

none

D. Current Postdoctoral Fellows, Research Scientists, and Jr. Faculty

Janet Stiernberg, Ph.D.	Adjunct Assistant Professor in Human Biological Chemistry and Genetics, Successful PI on Wound Healing Project, NIH funded SBIR grants to study cellular antimicrobial activity of the thrombin peptide TP508 and its effect on chronic wound healing and cartilage repair.
Roger Crowther, Ph.D.	Adjuct Assistant Professor, Dr Crowther directs the Chrysalis BioTechnology Analytical Laboratory and oversees formulation and stability testing of TP508 products. PI on several Phase I/II SBIR NIH grants to study effects of TP508 in fresh fracture and other orthopedic applications.
Andrea Norfleet, Ph.D.	Preclinical Study Director. Dr. Norfleet is studying the mechanism of tissue repair stimulation by the TP508 peptide. Her initial projects involve identifying matrix and growth factor molecules that are stimulated early in tissue repair tissue by addition of TP508. In these studies she is using quantitative histology, immunocytochemistry, and *in situ* hybridization. She also obtained funding for a new SBIR project in vascular repair that demonstrated that TP508 may effectively reduce restenosis even in hypercholesterolemic rabbits.
Michael Kerheley, Ph.D.	Adjuct Assistant Professor, Group Director for BioDiscovery and Molecular Biology. Initial projects involve work on cloning the NPAR thrombin receptor and development of in vitro biological assays to test synthetic peptides for activity

related to tissue repair. Mike is also working on development of new technologies for tissue repair, modulation of infection and inflammation, and anti cancer applications

Mohammad Saeed Postdoctoral, BioDiscovery and Molecular Biology, focusing on receptor cloning projects. Recently, Mohammad has used the yeast-2 hybrid system to identify a family of proteins that bind to thrombin and thrombin peptides. He has also constructed expression vectors which can be tagged or expressed with GFP to study effects of TP508 expression in cells.

MEMBERSHIP IN SCIENTIFIC SOCIETIES:
American Society for Cell Biology
The Wound Healing Society
American Diabetes Association (professional)
European Academy of Science

HONORS:

Research Career Development Award, National Cancer Institute (1982-87).
Distinguished Alumni (Albertson College of Idaho, 1998).

ADDITIONAL INFORMATION

Invited Seminars, Symposia and Special Presentations

1978 "Proteases and Cell Proliferation." Panel Discussion ICN-UCLA Winter Symposium (March, Keystone, Colorado)

1980 "Relationship Between Cell Surface Receptors and Cytoplasmic Microtubules." International Symposium on Fundamental Mechanisms in Human Cancer Immunology. (Oct. 27, Galveston, TX).

1980 "Initiation of Cell Division by Thrombin-Receptor Interaction" UTMB Cancer Center Seminar Series (Sept. 16).

1981 "Surface Receptors and Cytoskeletal Interactions in Control of Normal and Neoplastic Cell Proliferation" UTMB Research Conference - Mini Symposium on Role of Cell Membranes in Control of Metabolism and Cell Behavior (June 23, Galveston, TX),

1981 "Preclustering of Thrombin Receptors and Their Interaction With Cytoplasmic Microtubules: Possible Role in Growth Regulation." Division of Endocrinology Research Seminar, The University of Texas Medical School at Houston (Houston, TX, Oct. 29).

1981 Chair, Platform Session on Receptor Mediated Endocytosis. American Society for Cell Biology (Nov. 10, Anaheim, California),

1982 "The Role of Microtubule Alterations in Initiation of DNA Synthesis" Federation of North Texas Area Universities 5th Annual Molecular Biology Symposium (May 21, Denton, Texas).

1982 "Role of Surface Receptors and Transmembrane Signaling in Initiation of Cell Proliferation" Department of Pharmacology Research Seminar, The University of Texas Medical Branch, Galveston, Texas, (Nov. 5)

1983 "Cell Surface, Receptors, Cytoskeleton and Receptor-Cytoskeletal Interactions." Two week lecture series - University of Puerto Rico, Rio Piedras, San Juan Puerto, Rico (Oct. 23-Nov. 1).

1984 "Mini symposia on Cellular Signal Transduction with Hormones, Mitogenesis and Oncogenes," American Society for Cell Biology (Nov. 13, Kansas City).

1984 "Microtubule Involvement in Initiation of Cell Proliferation" New York Academy of Sciences Conference on Dynamic Aspects of Microtubule Biology, (Dec. 3-6).

1985 "Thrombin Stimulated Phosphoinositide Metabolism Appears Necessary for Thrombin Mitogenesis," 69th Annual meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA (April 21-26).

1985 "Double Lock Pathways Stimulated in Mitogenesis," Xth Congress of the International Society of Thrombosis and Haemostasis, San Diego, CA (July 15-18).

1985 "Role of Phosphoinositide Turnover in Thrombin Mitogenesis," 13th International Congress of Biochemistry -Amsterdam, The Netherlands (August 25-30).

1985 "Thrombin Receptor Occupancy Initiates Transient Increase in cAMP Levels in Mitogenically Responsive Hamster (NIL) Fibroblasts," New York Academy of Sciences, Conference on Bioregulatory Functions of Thrombin New York, NY (Feb. 5-7).

1985 Invited Seminar (International) "Thrombin receptors and transmembrane signals in regulation of cell proliferation" Centre de Biochimie, Seminar Program, Parc Valrose, Nice France (Sept. 1-4).

1986 International Workshop Organizer on Proteases and Biological Control. UCLA Symposium on Proteases, Park City, UT (Feb. 12).

1986 UTMB Representative, Special Conference on Academic-Industrial Interaction, Fisher Scientific Group, Hotel Del Coronado, San Diego, CA (July 10-13).

1986 "Modulation of Thrombin - Receptor Interaction in Cultured Neurofibroma and Neurosarcoma Cells," Texas Neurofibromatosis Foundation, Semi-annual meeting, Smithville, TX (Sept. 5).

1986 "Thrombin Peptide Interacts with High-Affinity Thrombin Receptors Initiating Part of the Proliferative Signal," Mini symposium on "Extracellular Proteases in Development and Neoplasia," at the 26th

Annual meeting of the American Society for Cell Biology, Washington, DC (Dec. 7-11).

1987 "Thrombin Stimulation of Proliferation: Role of Receptors, Cytoskeleton and Transmembrane Signals," Seminar-Department of Cell Biology and Anatomy, University of Alabama, Birmingham, AL (Feb. 18-20).

1988 "Thrombin Peptides Enhance Wound Closure and Increase Breaking Strength-Wound Healing Project Review." Monsanto Corporation Chesterfield, MO (March, 1988).

1988 "Mechanisms Involved in Thrombin Mitogenesis," Gordon Research Conference Speaker - Plymouth, NH (June 13-17).

1988 "Use of Synthetic Peptides as Probes for Receptor Ligand Interactions, Second Messenger Function and *in vivo* Modification of Biological Responses." Milligen Biosearch - National Frontiers in Molecular Biology Seminar Series.

1988 Invited Guest Speaker "Thrombin Receptors and Transmembrane Signals in Regulation of Cell Proliferation" Molecular Biology Seminar Series - University of Kansas, Lawrence KS (Feb. 3).

1989 "Thrombin and Synthetic Peptides in Wound Healing," Homecoming Address, The University of Texas Medical Branch, Galveston, TX (March 31).

1989 "Wound Healing Project Review - Research Alert." Monsanto Corporation, Chesterfield, MO (June 19-20).

1989 "Thrombin Peptides as Wound Healing Agents: Perspectives, Potential Efficacy, and Marketability," Monsanto Corporation - J.D. Searle and Company, Skokie, IL (August 30-31).

1990 "Thrombin and Thrombin Receptor Activating Peptides in Regulating Cell Proliferation *In Vitro and In Vivo*," University of Vermont Graduate Program Lecture Series in Cell and Molecular Biology, Burlington, VT (March 3-6).

1991 "Thrombin Peptides Promote Healing of Wounds in Steroid-Treated Rats." First International Meeting of the Wound Healing Society January 1991, Galveston, TX.

1991 "Synthetic Thrombin Peptides as Mediators of Cellular Processes *in vitro* and *in vivo*." Winter Neuropeptide Conference, Breckenridge CO, (February, 1991).

1991 "Postclotting Effects of Thrombin and Synthetic Thrombin Peptides: Potential Role in Wound Healing and Inflammation" Microbiology Seminar UTMB (May 1991).

1992 "Discovering Thrombin's Regulatory Diversity: Role of Thrombin and Thrombin Receptors in Cell Proliferation, Inflammatory Responses, and

Wound Healing." Faculty Research Colloquium: The University of Texas Medical Branch, (Jan. 27).

1992 "Research Update: Use of Synthetic Thrombin Peptides in Acceleration of Wound Healing." Johnson & Johnson Medical Inc., Dallas, TX (March 3-4).

1992 "Acceleration of Wound Healing and Thrombin Postclotting Cellular Activities *in vivo* using Synthetic Thrombin Receptor Activating Peptides" Somatix Therapy Corporation Seminar: Somatix Corp. Alameda CA. (April 24).

1992 "Role of Thrombin and Thrombin Receptors in Cell Proliferation, Inflammatory responses, and Wound Healing" Creative BioMolecules, Boston MA. (April 30).

1992 "Role of Thrombin and Synthetic Thrombin Receptor-Activating Peptides in Stimulation of Wound Healing, Inflammation, and Angiogenesis" Biogen Research Seminar, Boston, MA (August 6).

1992 "Stimulation of Wound Healing and Cellular Responses by Thrombin and Receptor Activating Thrombin Peptides" FASEB Conference on Structure and Function of Thrombin. Vermont (August 8-14).

1992 "Use of Synthetic Thrombin Peptides in Wound Healing." Research Update, Johnson & Johnson Medical Inc., Biopolymer Group, Stirling University, Stirling, U.K, (August 24).

1992 Delegate, 2nd European Tissue Repair Society Meeting, Malmo, Sweeden, (August 24-27). Johnson & Johnson Consultant

1993 Invited Research Seminar "Thrombin and Thrombin Peptides as Mediators of Inflammation and Tissue Repair" University of Houston, Biochemistry Department (March).

1993 State of the Art Lecture, "Role of Thrombin and Thrombin Peptides in Tissue Repair" International Congress of Thrombosis and Hemostasis, New York (July 3-12).

1993 "Efficacy of TRAP-508 in enhancing healing of incisional and open wounds in animal models" Spectrum Consumer Products, Houston TX (September 1993).

1994 "Effect of thrombin and thrombin peptides on corneal wound healing" Association for Research in vision and Ophthalmology, St. Petersburg Florida, (May 1994).

1994 Seminar, Thrombin Peptide Technology Update, Ventures Medical-Houston, TX (June 1994).

1994 Session Chair, "Thrombin and Cellular Systems" at the Fourth International Biennial Meeting on Blood Coagulation and Platelet Biology, "Thrombin functions and new Prospects in Antithrombotic therapy", Megeve, France, September 11-15, 1994.

1994 State of the Art Lecture, "Role of thrombin and thrombin peptides in initiation of inflammation and tissue repair" at the Fourth International Biennial Meeting on Blood Coagulation and Platelet Biology, Megeve, France, September 13, 1994.

1994 Invited International Seminar: "Role of thrombin and synthetic thrombin peptides in Inflammation and Wound Healing" University of Siena, Siena Italy, September 19, 1994

1995 Invited Seminar: "Effects of Thrombin and Synthetic Thrombin Peptides in Wound Healing" Cardiovascular Seminar Series, Sealy Center for Molecular Cardiology, UTMB, Galveston, TX.

1995 Discussant: FASEB Summer Conference on "Thrombin Structure and Function" Copper Mountain Colorado (August 1995).

1997 Seminar-Presentation: Thrombin peptides in wound healing. Biersdorf, AG, Hamburg, Germany, (January 10, 1997).

1997 Seminar-Presentations, "Thrombin Peptides in Wound Healing." Zurich Switzerland, Dr. Raphael Levi Feb. 13, 1997, and, Wuppertal, Germany, Bayer, AG. Feb. 14, 1997.

1997 Presentation, Bayer Biologics, New Haven, CT. "Thrombin and thrombin peptides in tissue repair" May 27, 1997.

1997 Presentation, US Surgical, New Haven CT., "Thrombin Peptide TP508 in soft and hard tissues: Potential therapeutic." May 28, 1997.

1997 Attendee: XVI Congress of the International Society on thrombosis and Haemostasis, Florence, Italy. June 4-11, 1997.

1997 Third FASEB Summer Conference on Thrombin, Saxon River Vermont. Meeting discussant - Presenter "Taking technology to market to support basic science research" August 9-13, 1997.

1997 Presentation: "Thrombin Peptide Use in Hard Tissue - Orthopedic Tissue Repair" OrthoLogic, Inc. Phoenix, AZ. October 13, 1997.

1997 Invited Seminar: Trinity University, SanAntonio, TX "Thrombin and Thrombin Peptides in Inflammation and Tissue Repair" Departments of Biology and Biochemistry October 20, 1997

1997 SBIR Workshop Presentation: "Opportunities to support basic science research using technology transfer and SBIR funding: Chrysalis BioTechnology, Inc. A Case Study" Universtiy of Texas Medical Branch health Science Center, Houston, TX. November 14, 1997.

1998 Keystone Winter Symposium, "Tissue Repair Mechanisms", Cooper Mountain, Colorado, January 10-14, 1998.

1998 Presentation to Drug Division, FDA "Use of Thrombin Peptide, TP508, in Surgical and Chronic Wound Healing, Pre-IND meeting. January, 20-21, 1998. Washington D.C.

1998 Invited Presentation. Arterial-Vascular Engineering (AVE), "Potential application of thrombin peptides for prevention of restenosis." January 29, 1998.

1998 Invited Presentation. Medtronic, Mineapolis Minn. "Potential application of thrombin peptides for prevention of restenosis." February 18, 1998.

1998 Invited Presentation. Medici Medical Technologies (The Edge Group), "Potential application of thrombin peptides for prevention of restenosis." February 20, 1998, Houston, TX.

1998 Invited Presentation. Guidant, San Francisco CA, "Potential application of thrombin peptides for prevention of restenosis." March 8-9, 1998.

1998 Seminar, UTMB Tissue Engineering Group, Pharmacology Conf. Rm., Synthetic peptides in Tissue Repair, Galveston, TX. March 10, 1998.

1998 OrthoLogic, Tempe, Arizona. "Use of Hyaluronic Acid as a vehicle for delivery of thrombin peptide, TP508." April 23, 1998.

1998 FDA presentation "Osteon" device for accelerated healing of fresh fracture. Presentation for device vs. drug determination for use of TP508 in orthopedic applications. April 29, 1998.

1998 Invited Seminar. "Use of TP508 for Interventional Cardiology" MIT, Cambridge Mass. Division of Cardiology. June 16, 1998.

1998 Wound Healing Society Annual Meeting, Oral Presentation. Thrombin peptide, TP508, stimulates wound healing through a non-proteolytic mechanism. Salt Lake City, Utah, June 20, 1998.

1998 OrthoLogic, Tempe, Arizona. Seminar, "Drug use of TP508 to accelerate fresh fracture healing." June 22, 1998.

1998 OrthoLogic, Tempe, Arizona. Meeting September 1, 1999

1998 Invited Cardiovascular Presentations, Guidant Corporation and AVE, September 9 and 10, 1998, California

1998 Invited Presentation, "Thrombin peptide TP508 use in soft and hard tissue repair." Trauma, Infection, and Repair Symposium, Galveston TX September 16,1998

1998 Invited Presentation, Washington DC "Effect of TP508 on neointima formation following angioplasty. AVE meeting with MIT collaborators. October 8,1998.

1998 Presentation and Discussions, UCSF. "Potential use of TP508 in spine fusion" November 12, 1998

1999 Invited Presentation. "New developments in Wound Healing with Chrysalin™ peptide TP508" 3M Corporation, Minneapolis, Minn. January 5, 1999.

1999 Invited Symposium Speaker Musculoskeletal Life Sciences Forum. "Tissue repair for the new millennium" Boston, Mass. January 27, 1999.

1999 Invited Presentation. "New developments in Wound Healing with Chrysalin™ peptide" Smith and Nephew, Tampa/St. Pettersburg, Florida. March 18, 1999.

1999 Invited Presentations (3). "New developments in Wound Healing with Chrysalin™ peptide" Baxter Hyland Immuno, Vienna Austria, Lohman Wound Care, Neuwied, Germany, and Smith Nephew, Hull, U.K. May 17-25, 1999.

1999 3rd Annual Biomaterials of the Future Conference, Medical Data International, SanFrancisco CA, "New advances in peptide technologies for repair of skin and bone" June 15, 1999.

1999 Symposium Speaker, Wound Healing Society, WOCN Joint Meeting and Educational Symposium, Therapeutic Possibilities for Problematic Wounds "Small Molecules for Wound Healing" Minneapolis Minn. June 20, 1999.

1999 Presentation to FDA, Washington DC, "Chrysalin™ for fracture healing in man" Pre-IND Meeting. July 15, 1999.

1999 Delegate, International Society for Thrombosis and Haemostasis Washington DC August 15-18,

1999 Attendee, Joint meeting of the European Tissue Repair Society and Wound Healing Society, Bordeaux France, August 24-28, 1999 .

1999 Presentation, "Thrombin peptide TP508 pre-clinical efficacy and Interim report on Diabetic Ulcer Trial DIAB001" Hollister, Chicago Illinois (September 2, 1999).

1999 Presentation, "Thrombin peptide TP508 pre-clinical efficacy and Interim report on Diabetic Ulcer Trial DIAB001" Healthpoint, San Antonio, TX (September 3, 1999)

1999 Presentation, "Thrombin peptide TP508 pre-clinical efficacy and Interim report on Diabetic Ulcer Trial DIAB001" Baxter Immuno Group Vienna Austria (September 8, 1999).

1999 Workshop on "Effects of thrombin and thrombin peptides on inflammatory cells and cytokines" Rome, IT (September 9-10, 1999).

1999 Civic Presentation "The Good Aspects of BioTechnology: Advances in wound care and bio engineering of tissues" Texas City Rotary Club

(November 2, 1999).

1999 Invited Seminar and Exploratory Discussion "Thrombin Peptides to promote repair of acute dermal, bone, and cardiovascular injuries: potential application to the Mars Mission" NASA, Houston, TX (November 9, 1999).

1999 Presentation, "Thrombin peptide TP508 pre-clinical efficacy and Interim report on Diabetic Ulcer Trial DIAB001" ConvaTec, Skillman, NJ (November 16-17).

2000 Presentation, "Thrombin peptide TP508 pre-clinical efficacy and Interim report on Diabetic Ulcer Trial DIAB001" Ross-Abbott, Coumbus Ohio (February 11, 2000).

2000 Invited Presentation "TP508 in Chronic Ulcers, Interim Data Diabetic Ulcer Trial DIAB001 and plans for international marketing" Abbott Laboratories (March 9, 2000).

2000 Co-Organizer and Speaker, 1st International Certosa de Pontignani Symposium: Thrombin and Thrombin Peptides in Inflammation and Tissue Repair. Siena, IT (May 13-16, 2000).

2000 Meeting and Discussions with companies:
Wound Healing Society Toronto, Canada (June 3-6,2000)

2000 Meeting and Discussions with companies: American Diabetes Association Meeting meet with clinical trial site coordinators San Antonio, TX (June 9-11,2000).

2000 Meeting and Discussions with companies: Direct Myocardial Revascularization, Washington DC (Separate meetings to set up collaborations to revascularize ischemic heart with Baylor and MicroMed Technologies), (June 21-23, 2000).

2000 Civic Presentation, "Chrysalis and Chrysalin ®, update on developing pharmeceutical companies in Texas" Representative Patricia Gray, Galveston, TX. (July 13,2000).

2000 Writing workshop (European Grant), Siena IT (August 16-22).

2000 Orthopedic TP508 Workshop, Sun Valley Idaho (August 30-September 2, 2000).

2000 Presentation, "Potential of TP508 in myocardial revascularization and inhibition of restenotic lesions" Abbott Laboratories Cardiovascular Development Group. (September 21, 2000).

2000 Participant, Tissue Repair Symposium, Virginia Commonwealth University, Richmond VA. (September 25-26, 2000).

2000 Invited Corporate Presentation (Delivered by D McWilliams) SouthWest BioVentures Conference, Moody Gardens (December 6, 2000).

2001 Thrombin Peptide Molecular Biology Symposium, Tremont House, Galveston TX (January 11-13, 2001).

2001 Presentation, "Effect of TP508 on porcine wounds and Othropedic update" (joint meeting with Chrysalis, Abbott, and OrthoLogic, Philadelphia, PA, March 12, 2001).

2001 Presentation, "TP508 interaction with NPAR, Background related to novelty of prior discoveries" U.S. Patent Office, Washington, DC. (June 5, 2001).

2001 Invited Speaker and Session Leader, 6th International Meeting on Angiogenesis: Basic Science and Clinical Developments. "Tissue repair stimulated by the angiogenic thrombin peptide, TP508" Crete, Greece (June 26-July 2nd, 2001).

2001 Invited Speaker, 3rd Annual Conference on Angiogenesis: Innovative Science and New Applications. "Thrombin Peptide TP508: An Angiogenic Factor that Accelerates both Dermal Wound Healing and Fracture Repair." Boston, MA (July 31, 2001).

2001 Delegate, European Tissue Repair Society Conference. Wales, UK (September 3-7, 2001).

2001 Investigators Meeting "Results of Phase II Trial Effect of TP508 on Diabetic Ulcers (Chrysalis DIAB001), Tremont House Hotel, Galveston, TX (September 8, 2001).

2001 Presentation, "Effects of TP508 on Distal Radius Fracture Phase I/II Trial (OrthoLogic)" FDA, Washington, DC (October 29, 2001).

2001 Invited Speaker & Roundtable Discussant, "Managing the Spinout Process: The Story of Chrysalis BioTechnology" SouthWest BioVenture Conference. Houston, TX (December 4-5, 2001).

2001 Four Poster Presentations, American Society for Cell Biology Annual Meeting, Washington DC (December 8-12, 2001).

BIBLIOGRAPHY

A. ARTICLES IN JOURNALS:

1. **Carney, D. H.** and Cunningham, D. D. Initiation of chick cell division by trypsin action at the cell surface. Nature 268: 602-666, 1977.

2. **Carney, D. H.**, Glenn, K. C. and Cunningham, D. D. Conditions which affect initiation of animal cell division by trypsin and thrombin. J. Cellular Physiol. 95:13-22, 1978.

3. Baker, J. B., Barsh, G. S., **Carney, D. H.** and Cunningham, D. D. Dexamethasone modulates the binding and action of epidermal growth factor in serum-free cell culture. Proc. Natl. Acad. Sci. USA 75:1882-1886, 1978.

4. **Carney, D. H.** and Cunningham, D. D. Cell surface action of thrombin is sufficient to initiate division of chick cells. Cell 14:811-823, 1978.

5. **Carney, D. H.** and Cunningham, D. D. Role of specific cell surface receptors in thrombin-stimulated cell division. Cell 15:1341-1349, 1978.

6. **Carney, D. H.** and Cunningham, D. D. Transmembrane action of thrombin initiates chick cell division. J. Supramol. Struct. 9:337-350, 1978.

7. **Carney, D. H.**, Glenn, K. C., Cunningham, D. D., Das, M., Fox, C. F. and Fenton, J. W., II. Photoaffinity labeling of a single receptor for alpha-thrombin on mouse embryo cells. J. Biol. Chem. 254:6244-6247, 1979.

8. Glenn, K. C., **Carney, D. H.**, Fenton, J. W., II and Cunningham, D. D. Thrombin active site regions required for fibroblast receptor binding and initiation of cell division. J. Biol. Chem. 255:6609-6616, 1980.

9. **Carney, D. H.** Visualization of thrombin receptors on mouse embryo fibroblasts using fluorescein-amine conjugated human-thrombin. J. Supramol. Struct. 13:467-478, 1980.

10. Crossin, K. L. and **Carney, D. H.** Evidence that microtubule depolymerization early in the cell cycle is sufficient to initiate DNA synthesis. Cell 23:61-71, 1981.

11. Crossin, K. L. and **Carney, D. H.** Microtubule stabilization by taxol inhibits initiation of DNA synthesis by thrombin and epidermal growth factor. Cell 27:341-350, 1981.

12. **Carney, D. H.** and Bergmann, J. S. ^{125}I-thrombin binds to clustered receptors on noncoated regions of mouse embryo cell surfaces. J. Cell Biol. 95:697-703, 1982.

13. Bergmann, J. S. and **Carney, D. H.** Receptor-bound thrombin is not internalized through coated pits in mouse embryo cells. J. Cell. Biochem. 20:805-817, 1982.

14. Stiernberg, J., LaBelle, E. F. and **Carney, D. H.** Demonstration of a late amiloride-sensitive event as a necessary step in initiation of DNA synthesis by thrombin. J. Cell. Physiol. 117:272-281, 1983.

15. **Carney, D. H.** Immunofluorescent visualization of specifically bound thrombin reveals cellular heterogeneity in number and density of thrombin receptors. J. Cell. Physiol. 117:297-303, 1983.

16. Thompson, W. C., Asai, D. J. and **Carney, D. H.** Heterogeneity among microtubules of the cytoplasmic microtubule complex detected by a monoclonal antibody to alpha tubulin. J. Cell. Biol. 98:1017-1025, 1984.

17. Stiernberg, J., **Carney, D. H.**, Fenton, J. W., II, and LaBelle, E. F. Initiation of DNA synthesis by human thrombin: Relationships between receptor binding, enzymic activity and stimulation of $^{86}Rb^{+}$ influx. J. Cell. Physiol. 120:289-295, 1984.

18. **Carney, D. H.**, Stiernberg, J. and Fenton, J. W., II. Initiation of proliferative events by human-thrombin requires both receptor binding and enzymic activity. J. Cell. Biochem. 26:181-195, 1984.

19. **Carney, D. H.**, Scott, D. L. and Gordon E. A. Phosphoinositides in mitogenesis: Neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. Cell 42:479-488, 1985.

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US005500412A

United States Patent [19]

Carney et al.

[11] Patent Number: 5,500,412

[45] Date of Patent: Mar. 19, 1996

[54] **THROMBIN DERIVED POLYPEPTIDES; COMPOSITIONS AND METHODS FOR USE**

[76] Inventors: **Darrell H. Carney**, 5020 Cedar Creek, Dickinson, Tex. 77539; **Kevin C. Glenn**, 12480 Glengate Dr., Maryland Heights, Mo. 63043

[21] Appl. No.: **7,173**

[22] Filed: **Jan. 21, 1993**

Related U.S. Application Data

[62] Division of Ser. No. 925,201, Oct. 31, 1986, Pat. No. 5,352,664.

[51] **Int. Cl.**[6] **A61K 38/16**

[52] **U.S. Cl.** **514/13**; 530/326

[58] **Field of Search** 514/12–17; 530/324–330

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Primary Examiner—Jill Warden
Assistant Examiner—David Lukton
Attorney, Agent, or Firm—Arnold, White & Durkee

[57] **ABSTRACT**

Thrombin is now known to mediate a number of potent biological effects on cells bearing high-affinity thrombin receptors. These effects depend, at least in part, upon receptor occupancy signals generated by thrombin's interaction with the high affinity thrombin receptor. The present inventors have formulated synthetic thrombin derivatives capable of selectively stimulating or inhibiting thrombin receptor occupancy signals. The stimulatory thrombin derivatives to bind to cell surface thrombin receptors and stimulate DNA synthesis in cells treated with non-mitogenic concentrations of alpha-thrombin or phorbol myristate acetate. Thus, these peptides, which have both a thrombin receptor binding domain and a segment of amino acids with a sequence common to a number of serine proteases, appear to generate receptor-occupancy dependent mitogenic signals. The inhibitory derivatives, which have no serine esterase conserved amino acid sequences bind to thrombin receptors without generating receptor-occupancy dependent mitogenic signals. This invention describes the peptides and methods for using them to promote cell growth and wound healing or to inhibit scar formation, tissue adhesions, and tumor metastasis and angiogenesis.

6 Claims, 6 Drawing Sheets

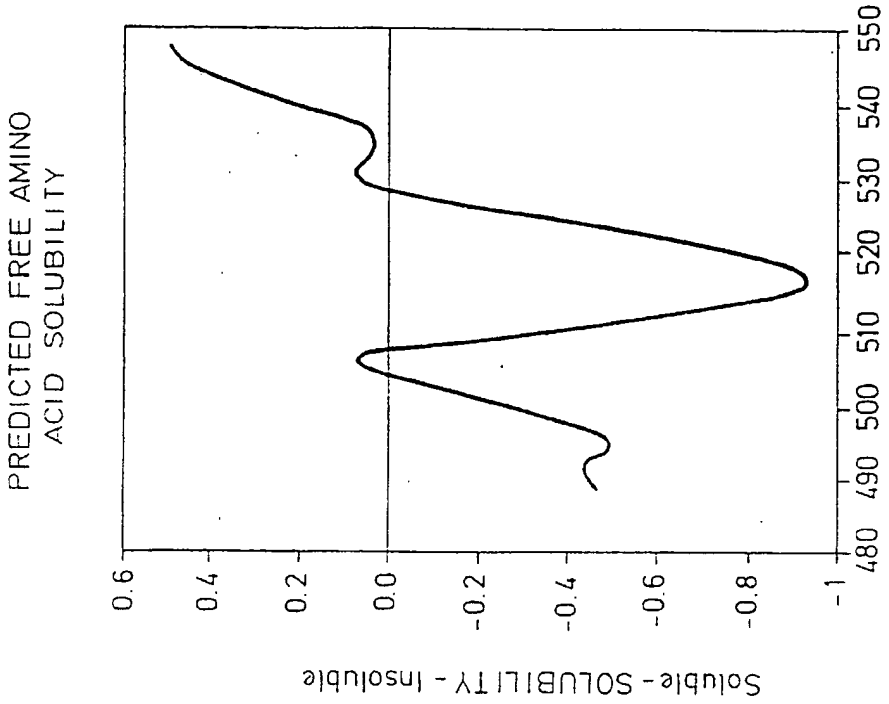

PREDICTED FREE AMINO
ACID SOLUBILITY
Soluble - SOLUBILITY - Insoluble
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1
480 490 500 510 520 530 540 550
RESIDUE NUMBER

Fig-1B

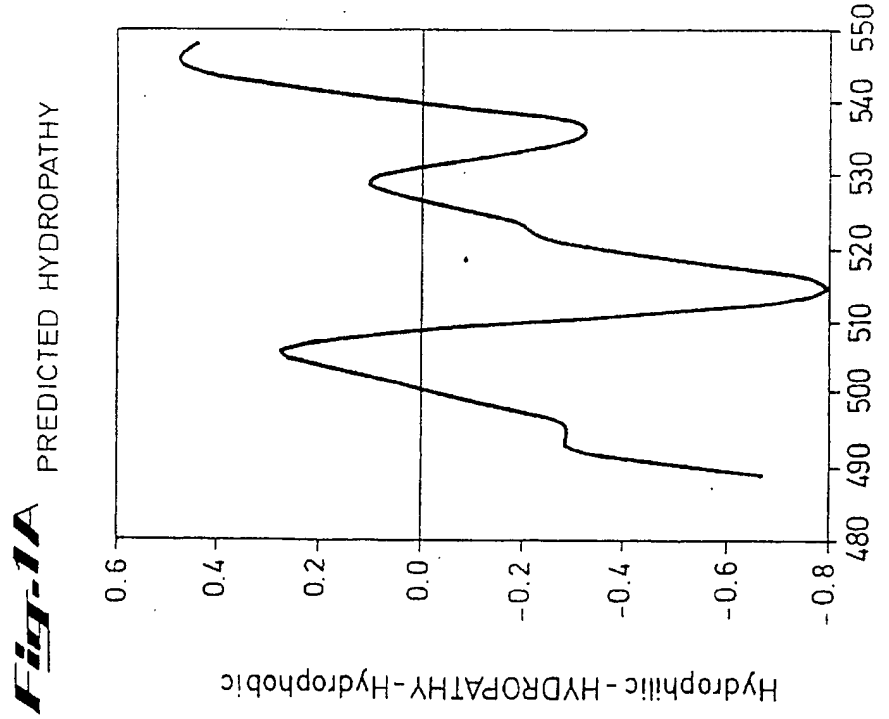

PREDICTED HYDROPATHY
Hydrophilic - HYDROPATHY - Hydrophobic
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
480 490 500 510 520 530 540 550
RESIDUE NUMBER

Fig-1A

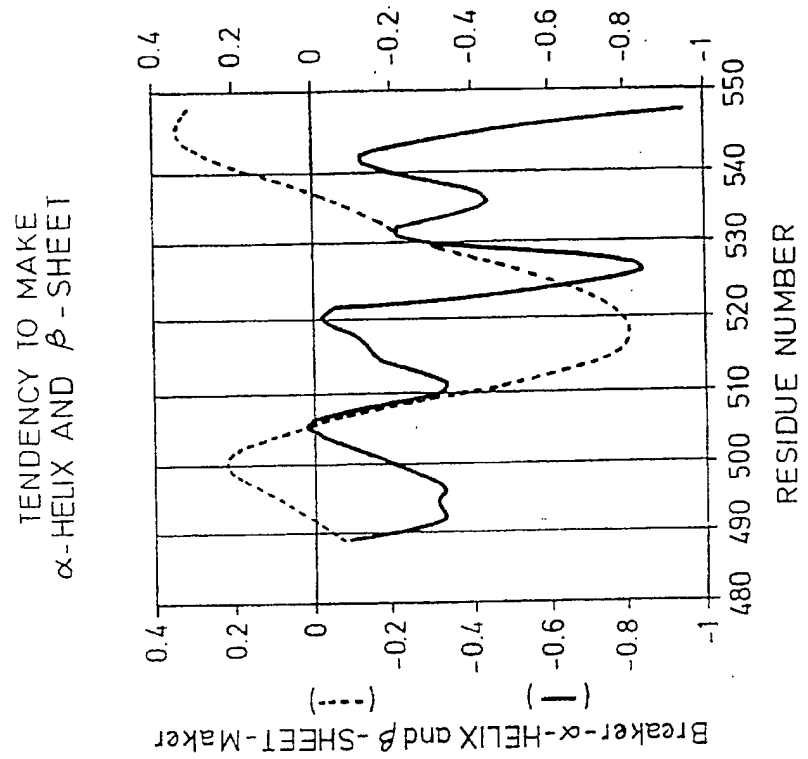

TENDENCY TO MAKE
α-HELIX AND β-SHEET
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
480 490 500 510 520 530 540 550
RESIDUE NUMBER
Breaker-α-HELIX and β-SHEET-Maker
(----)
(—)

Fig-1D

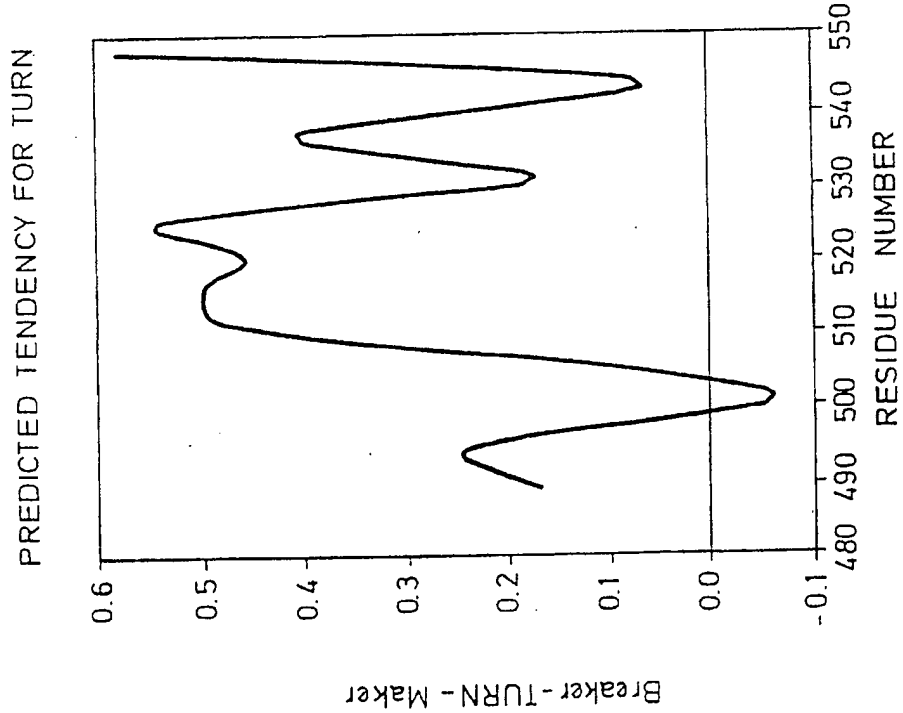

PREDICTED TENDENCY FOR TURN
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
480 490 500 510 520 530 540 550
RESIDUE NUMBER
Breaker-TURN-Maker

Fig-1C

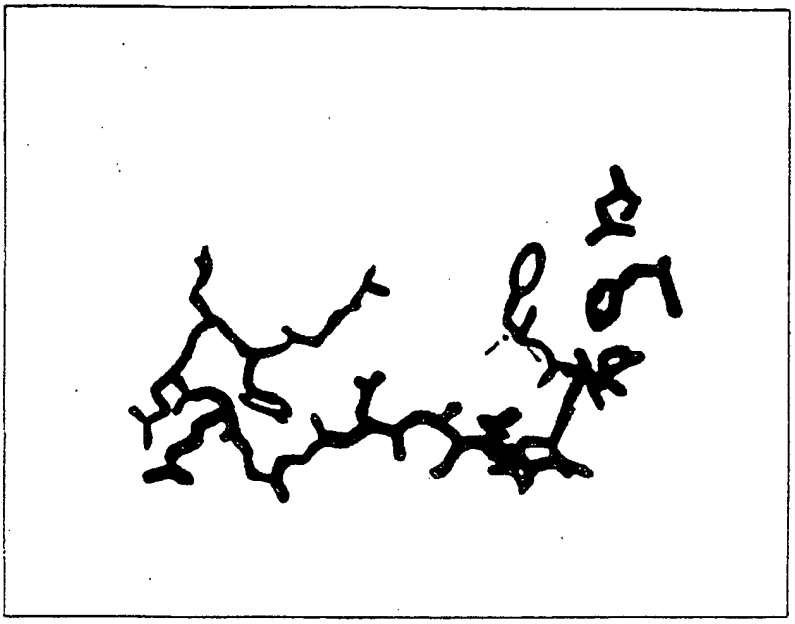


Fig.2B

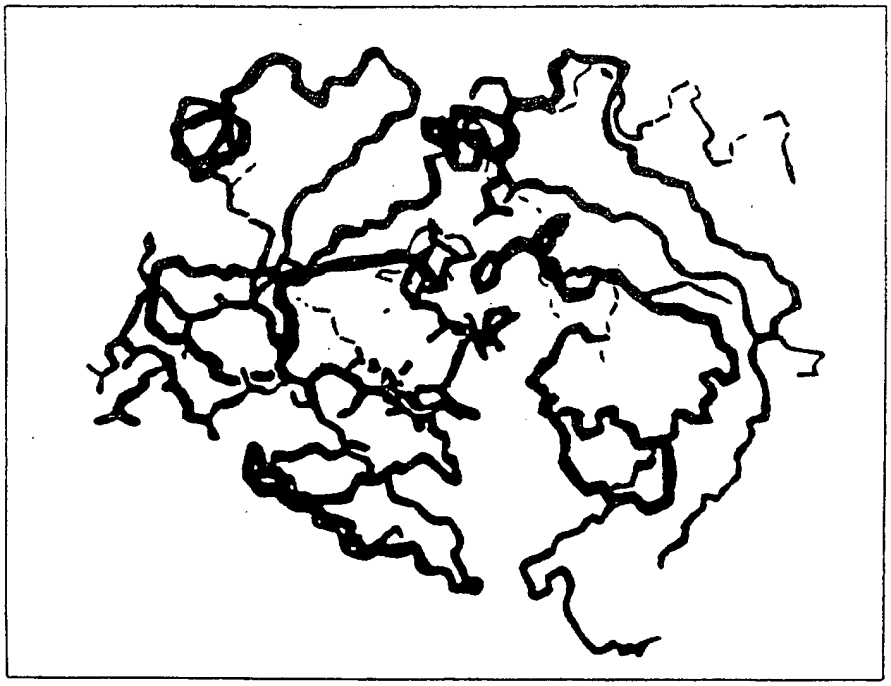


Fig.2A

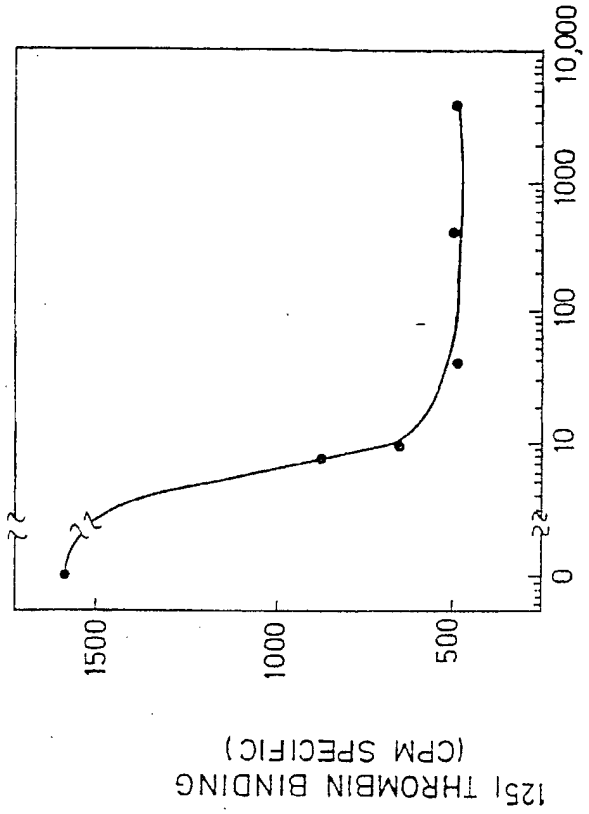

125I THROMBIN BINDING
(CPM SPECIFIC)
1500
1000
500
0
10
100
1000
10,000
p 508-530 (nM)

Fig. 3

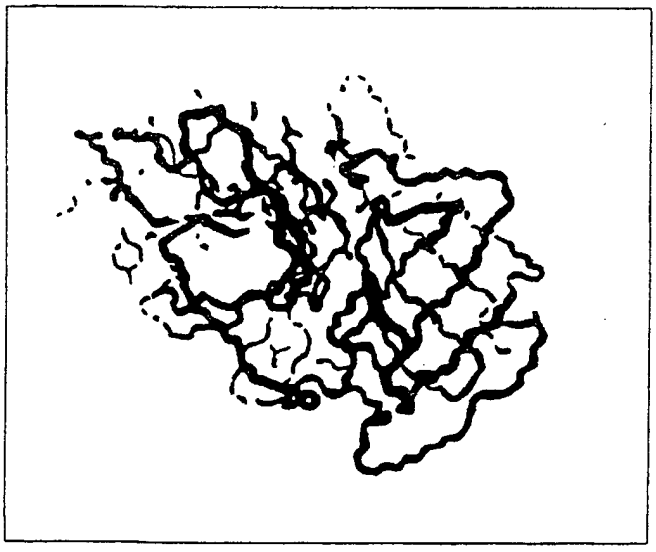


Fig. 2C

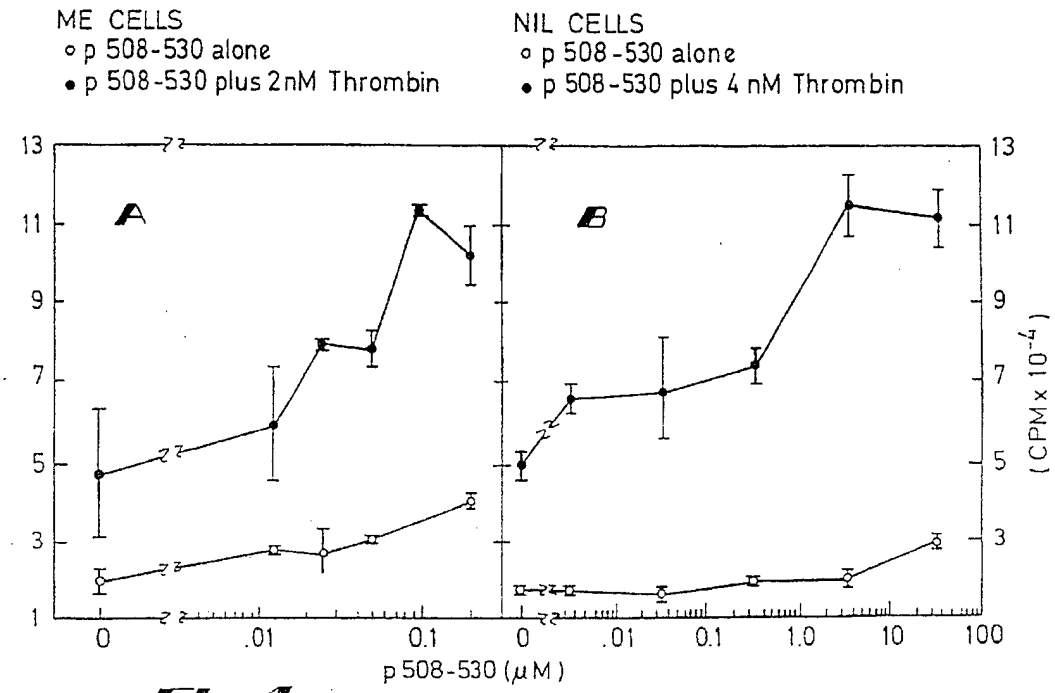

ME CELLS
o p 508-530 alone
• p 508-530 plus 2nM Thrombin
NIL CELLS
o p 508-530 alone
• p 508-530 plus 4 nM Thrombin
A
B
13
11
9
7
5
3
1
0
.01
0.1
1.0
10
100
p 508-530 (μM)
(CPM x 10⁻⁴)

Fig. 4

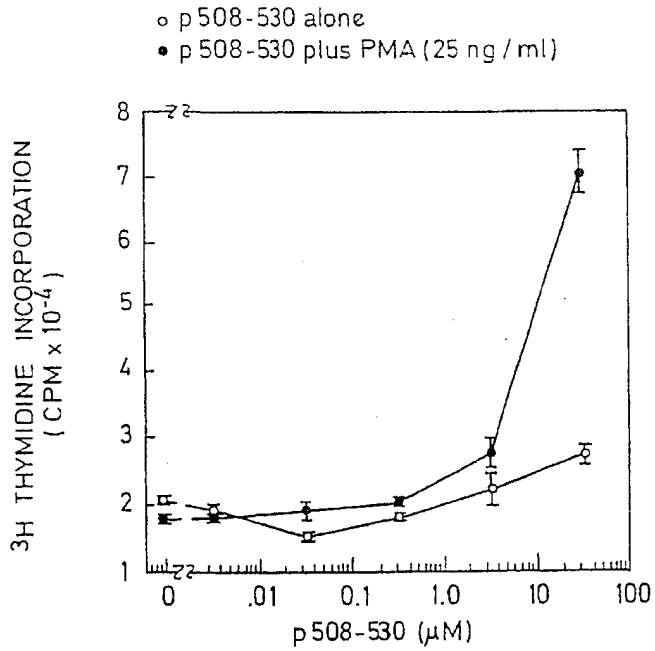
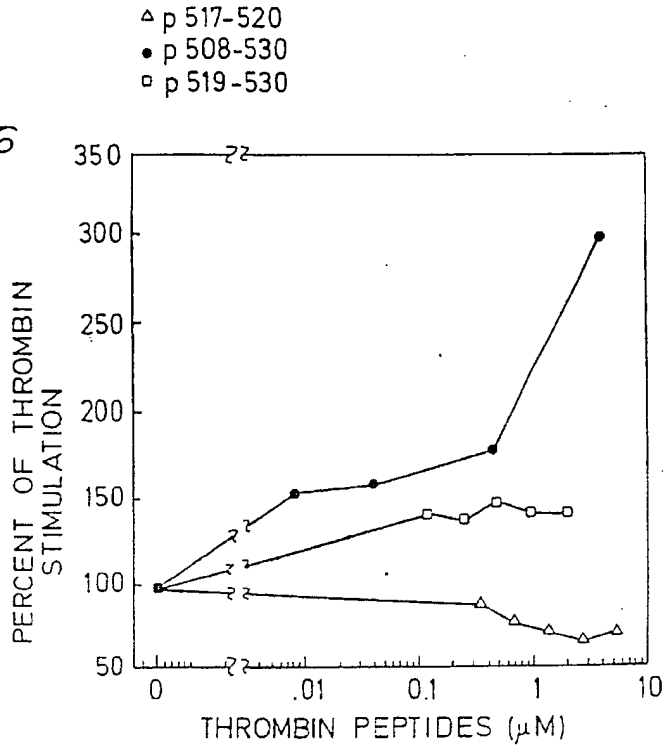

o p 508-530 alone
• p 508-530 plus PMA (25 ng / ml)
3H THYMIDINE INCORPORATION
(CPM x 10⁻⁴)
8
7
6
5
4
3
2
1
0
.01
0.1
1.0
10
100
p 508-530 (μM)

Fig. 5

Fig. 6


△ p 517-520
• p 508-530
▫ p 519-530
PERCENT OF THROMBIN STIMULATION
350
300
250
200
150
100
50
0
.01
0.1
1
10
THROMBIN PEPTIDES (μM)

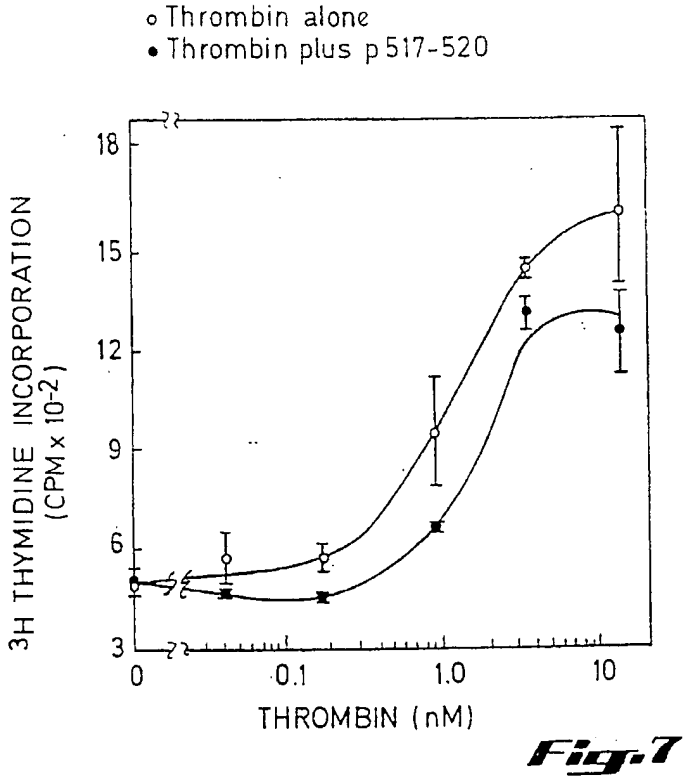

○ Thrombin alone
• Thrombin plus p 517-520
^{3}H THYMIDINE INCORPORATION (CPM x 10^{-2})
18
15
12
9
6
3
0
0.1
1.0
10
THROMBIN (nM)

Fig. 7

THROMBIN DERIVED POLYPEPTIDES; COMPOSITIONS AND METHODS FOR USE

The government owns rights in the present invention pursuant to grant Nos. CA00805 and AM25807 from the National Institutes of Health.

This is a divisional of application Ser. No. 06/925,201 filed Oct. 31, 1986, now U.S. Pat. No. 5,352,664.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention is directed to chemical compounds and methods useful in the regulation of thrombin receptor mediated cell stimulation. More specifically, the invention is directed to prothrombin-derived peptides and methods which employ such peptides for promoting wound healing and inhibiting scar formation, tissue adhesions, blood coagulation, tumor angiogenesis, tumor metastasis and pulmonary edema.

2. Description of the Related Art

Human alpha-thrombin appears to have growth-promoting activity for a wide variety of cells from various tissues. For example, alpha-thrombin has been shown to initiate proliferation of fibroblastic cells in culture without addition of serum or other purified growth factors, to synergize with epidermal growth factor in certain hamster fibroblasts and human endothelial cells, and to initiate cell division or DNA synthesis in mammalian lens epithelial and spleen cells. Yet, the use of thrombin as a growth factor and its potential importance to wound healing has not been widely acclaimed. In part, this may be due to the complexity of thrombin's involvement with coagulation, platelet activation, and initiation of cell proliferation as well as to the complex regulation of thrombin and thrombin-like molecules by serum protease inhibitors and by cell-released protease nexins. This complexity and high degree of physiologic regulation, however, supports the potential importance of this initiation pathway in wound healing.

Thrombin may also play a role in metastasis and angiogenesis of tumors. Generally, for a tumor to grow larger than a few millimeters in diameter, vascular endothelium must proliferate and form vesicle walls to provide circulation and nutrients to the cells inside of the tumor mass. Thrombin likely potentiates this process by virtue of its ability to induce proliferation of endothelial cells. In addition, thrombin has been shown to disrupt the normal intercellular endothelial cell contacts important in preventing cells and plasma factors from escaping or entering the microvasculature. The present hypothesis that thrombin may increase metastasis by disrupting these contacts is supported by studies demonstrating a correlation between decreased levels of anti-thrombin III (which removes thrombin and other proteases from plasma) and increased tumor metastasis.

Various studies have led the present inventors to conclude that high-affinity cell surface thrombin receptors (See Carney and Cunningham,. *Cell* 15:1341, 1978) may be involved in tumor metastasis and anglogenesis. For example, studies have indicated that thrombin receptors can serve as binding sites for tissue plasminogen activator, a molecule secreted from metastatic tumor cells. Moreover, other studies demonstrate the involvement of tissue plasminogen activator in metastasis and angiogenesis. Thus, many of the effects of plasminogen activator may be mediated through its interaction with the cell surface thrombin receptor. It is therefore proposed that stimulation of the thrombin receptor serves to promote tumor metastases, while inhibition of the receptor will decrease metastatic activity.

Thrombin has also been shown to cause changes in the structure and function of cells which make up the endothelial vasculature. These studies suggest that thrombin may play a central role in the development of pulmonary edema as well as edema of other tissues. For example, thrombin has been shown to increase permeability of endothelial cell monolayers to macromolecules, to increase arterial pressure and pulmonary vascular resistance, to induce smooth muscle contraction, and to increase transcapillary fluid filtration. All of these effects may be mediated by thrombin's interaction with cell surface thrombin receptors.

A number of recent studies have attempted to elucidate the mechanisms for thrombin-mediated cell stimulation. These studies have indicated to the present inventors that initiation of cell proliferation by thrombin requires two signals. The first signal appears to be dependent upon binding of the thrombin molecule to the high affinity cell surface thrombin receptor, while the second signal results from the enzymic activity of the thrombin molecule. Thus, unlike alpha-thrombin, neither DIP-alpha-thrombin (a proteolytically inactive thrombin derivative that retains receptor-binding activity) nor gamma-thrombin (an esterolytically active, but non-binding thrombin derivative) can initiate DNA synthesis or cell division. However, simultaneous addition of these two non-mitogenic thrombin derivatives initiates a level of DNA synthesis and cell division comparable to that initiated by alpha-thrombin.

These same thrombin derivatives have been used to distinguish intracellular events stimulated by high-affinity thrombin receptor occupancy from those resulting from proteolytic cleavage. Alpha-thrombin and gamma-thrombin both stimulate Na^+/K^+ ATPase activity, phosphoinositol turnover, and Ca^{2+} metabolism, whereas DIP-alpha-thrombin does not. Thus, these events are attributable to thrombin's enzymic activity, not to receptor occupancy. Furthermore, these signals (the release of diacylglycerol and inositol triphosphate to cause Ca^{2+} mobilization) may in turn activate protein kinase C. Accordingly, it has been shown that phorbol myristate acetate (PMA), which activates protein kinase C, can substitute for enzymically active gamma-thrombin and initiate cell division in the presence of receptor saturating levels of DIP-alpha-thrombin or monoclonal antibody to the thrombin receptor. Thus, the requirements for enzymically active thrombin may indirectly relate to its activation of protein kinase C.

The precise signals generated by high-affinity interaction of thrombin with its receptor have been more difficult to define. However, it has recently been shown that DIP-alpha-thrombin stimulates a transient increase in intracellular cAMP. In contrast to ion fluxes and phosphoinositide turnover, cAMP levels are maximally stimulated by DIP-alpha-thrombin but are not stimulated by gamma-thrombin. Attempts to replace DIP-alpha-thrombin with cAMP analogs, however, have been unsuccessful. Therefore, it is possible that thrombin receptor occupancy produces a number of signals in addition to changes in cAMP levels.

One problem associated with the clinical application of thrombin directly to achieve such benefits is its susceptability to protease inhibitors by serum anti-thrombins. Such problems have heretofore prevented the use of thrombin in the clinic and has led the present inventors to identify smaller thrombin-active and thrombin antagonistic polypeptides which are not sensitive to the inhibitory effects of thrombin inhibitors.

The present invention provides for a number of smaller polypeptides which have been tailored to interact with the thrombin receptor to selectively stimulate or inhibit thrombin receptor occupancy related signals. It is believed that these polypeptides will prove to be useful in a wide variety of clinical settings where successful recovery may be influenced by thrombin receptor-mediated events.

SUMMARY OF THE INVENTION

The present invention provides a number of thrombin derivatives and methods useful for stimulating cell proliferation and promoting wound healing as well as methods useful in inhibiting wound healing, scar tissue formation, formation of tissue adhesions, and tumor metastasis and angiogenesis. The invention is based on the discovery that one may formulate polypeptide thrombin derivatives, or their physiologically functional equivalents, which selectively inhibit the interaction of thrombin with its high-affinity receptor or which mimic the stimulatory effects of thrombin.

Accordingly, the present invention, in its most general and overall scope, relates to synthetic or naturally derived polypeptide agonists and antagonists of thrombin receptor mediated events. Both of these classes of agents possess a thrombin receptor binding domain which includes a segment of the polypeptide that is capable of selectively binding to the high-affinity thrombin receptor. This segment of the polypeptide includes a sequence of amino acids homologous to a tripeptide cell binding domain of fibronectin.

In addition to the thrombin receptor binding domain, the stimulatory (agonistic) polypeptides possess a sequence of amino acids having sequences derived from the N-terminal amino acids of a dodecapeptide previously shown to be highly conserved among serine proteases. However, the inhibitory polypeptides do not include these serine esterase-conserved sequences.

The present invention is disclosed in terms of a showing that in the presence of a non-mitogenic (ie, non-stimulatory) concentration of alpha-thrombin, gamma-thrombin, or PMA, the interaction between stimulatory polypeptides and cell surface thrombin receptors provides the cell with a signal to proliferate. However, no proliferative signal results when cell surface thrombin receptors interact with the inhibitory polypeptides. Instead, the cells become more refractory to subsequent treatment with the stimulatory polypeptides. This result is believed to occur because the inhibitory polypeptides, which are themselves incapable of generating a proliferative signal, block binding of the stimulatory polypeptides.

As indicated above, practice of the cell-stimulatory methods of the present invention requires the presence of a secondary signal, for example, in the form of non-mitogenic concentrations of alpha-thrombin, gamma-thrombin, or PMA in order to supply the cells with the low-affinity proteolytic cleavage signal. Accordingly, the invention provides for pharmaceutical compositions and methods to which these compounds have been added. However, those of skill in the art will recognize that when the invention is practiced in vivo, native alpha-thrombin endogenous to the host will typically be adequate to provide this secondary signal.

Because thrombin is involved in a number of bioregulatory effects, the present invention, which allows one to selectively promote and inhibit these effects, has a number of clinical applications. For example, the invention provides a number of polypeptides useful in promoting wound healing. For such applications, the invention provides a polypeptide derivative of thrombin (or a functional equivalent of such a derivative) which has a thrombin receptor binding domain as well as a domain with a serine esterase conserved sequence of at least 12 amino acids. The invention also provides a polypeptide compound of at least 23 L-amino acids which has both a thrombin receptor binding domain and a domain with a serine esterase conserved amino acid sequence.

In one embodiment, the invention provides for several polypeptides containing specific amino acid sequences, such as a polypeptide compound in which the thrombin receptor binding domain includes the L-amino acid sequence Arg-Gly-Asp-Ala together with the serine esterase conserved amino acid sequence, Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val. In a preferred embodiment, the polypeptide compound includes the L-amino acid sequence Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

The invention also provides for a pharmaceutical composition for promoting wound healing which includes of a therapeutically effective concentration of any of the compounds described above combined with a pharmaceutically acceptable excipient. Typically, such compositions include, for example, sufficient concentrations of the polypeptides to effect a stimulatory action on the thrombin receptor as demonstrated herein. Thus, such compositions should typically include sufficient concentrations to obtain levels of the polypeptides in the wound area which are shown in vitro to stimulate the receptor. When endogenous levels of a secondary signal are believed to be inadequate, compositions may be employed which further include the addition of a therapeutically effective concentration of alpha-thrombin or gamma-thrombin.

As used herein, a therapeutically effective concentration is defined as a concentration of the particular agent which provides a satisfactory increase in the rate of wound healing. Again, such concentrations are believed to correspond to levels sufficient to ellicit a stimulation of the thrombin receptor in vitro. However, it is believed that the compositions will prove most effective when the stimulatory (agonistic) polypeptides are present at a concentration of from 0.1 uM to 10 uM.

Furthermore, where alpha-thrombin or gamma-thrombin are also employed, concentrations of from 0.1 uM to 10 uM are considered effective. However, empirical methods as are known in the art may be employed for determinihg more precisely the proper therapeutic dose for a given composition administered in a particular manner.

In addition, methods are provided which employ thrombin agonists to promote wound healing. One such method includes applying to the wound a therapeutically effective amount of a polypeptide derivative of thrombin, or a physiologically functional equivalent thereof, which has both a thrombin receptor-binding domain and a domain having a serine esterase conserved amino acid sequence. In general, thrombin is applied in amount sufficient to achieve fibroblast stimulation and thereby stimulate tissue regeneration. In that such methods typically involve topical application to a wound, possible sytstemic toxicity is not believed to be a problem. Therefore, virtually any concentration may be employed. However, in a preferred embodiment, the wound is treated to achieve a range of approximately 1 ng/cm^2–10 ug/cm^2 of wound surface.

The invention further provides a method for promoting wound healing in which a therapeutically effective amount

of alpha-thrombin (1 ng/cm^2–10 ug/cm^2 of wound surface) or gamma-thrombin (1 ng/cm^2–10 ug/cm^2 of wound surface) is applied to the wound in conjunction with the foregoing thrombin derivatives. Of course, the specific polypeptides and pharmaceutical compositions provided by the invention may also be used in promoting wound healing. It is believed that these methods will be especially beneficial to patients involved in severe accidents (particularly burn patients), to those subjected to surgical procedures and to those with poor wound healing responses, such as aged and diabetic individuals.

Additional methods are provided for using the thrombin receptor inhibitory polypeptides. For example, the invention provides methods whereby scar tissue formation can be inhibited by administering to the wound or scar tissue, a therapeutically effective amount of a polypeptide derivative of thrombin, or a physiologically functional equivalent thereof, which has a thrombin receptor binding domain but does not have a serine esterase conserved sequence. Typically, such concentrations are adequate when sufficient to inhibit thrombin receptor mediated events. In a preferred embodiment, amounts ranging from 1 ng/cm^2–10 ug/cm^2 of wound surface are considered appropriate.

In a preferred embodiment, the polypeptide derivative of thrombin has the L-amino acid sequence Arg-Gly-Asp-Ala.

In general, these methods may be used in any situation where scar formation is undesirable, such as on burn patients or those subjected to opthalmic surgery. Moreover, the methods may also be of use in preventing keloidal scar formation. It is anticipated that spraying the wound with an aerosol spray will be a particularly sterile and efficacious manner of administering the polypeptide compound to the wounds of burn patients.

The inhibitory polypeptides should also prove useful in inhibiting the formation of tissue adhesions, defined as abnormal unions between body organs by formation of fibrous tissue. It is known that fibroblast proliferation is required for formation of such adhesions. Since alpha-thrombin is known to induce fibroblast proliferation, it follows that inhibition of thrombin-mediated mitogenesis by the peptides of the present invention could reduce adhesion formation. It is believed that administration of such inhibitory polypeptides to the surface of the affected organs will prove to be especially useful following certain surgical procedures, such as thoracic surgery, where gut adhesions often lead to postoperative complications.

It is further proposed that the inhibitory peptides will prove useful in the treatment of mammals with tumors to thereby inhibit tumor metastasis or anglogenesis. This view is supported by studies demonstrating that alpha-thrombin is able to disrupt normal inter-endothelial cell contacts important in preventing metastasis, as well as studies demonstrating that alpha-thrombin can induce the proliferation of endothelial cells required for angiogenesis. Accordingly, the invention provides a method whereby mammals with such tumors receive a therapeutically effective amount of a polypeptide derivative of thrombin, or a functional equivalent thereof, which has a thrombin receptor binding domain but does not have a serine esterase conserved sequence. While exact doses would need to be determined by empiracal methods known those skilled in the art, it is estimated that an amount sufficient to achieve a concentration of from 0.1 uM to 10 uM at the site to be treated is needed. Use of a polypeptide wherein the thrombin binding domain has an L-amino acid sequence Arg-Gly-Asp-Ala is specifically provided. It is contemplated that the polypeptides will be most efficacious in this regard when administered intravenously. However, other methods of administration will also likely prove to be effective.

In a most general embodiment, the invention provides for the use of inhibitory polypeptides to inhibit cell proliferation. This method encompasses, but is not limited to, situations in which one desires to inhibit cell proliferation in vitro. Of course, the inhibitory polypeptide, having a thrombin binding domain with the specific sequence Arg-Gly-Asp-Ala, may also be used as a general inhibitor of cell proliferation.

In another general embodiment, the invention comprises methods wherein the stimulatory polypeptides are used to potentiate cell growth. A polypeptide including the sequence Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val is specifically provided. This method encompasses, but is not limited to, situations wherein one wishes to potentiate cell growth in vitro. Such cell-stimulatory uses may be potentiated by further providing an effective amount of alpha-thrombin (0.1 ug/ml–10 ug/ml), gamma-thrombin (0.1 ug/ml–10 ug/ml) or phorbol myristate acetate (10 ng/ml–100 ng/ml) in conjunction with the stimulatory polypeptide.

GLOSSARY

For purposes of the present invention, a thrombin derivative is defined as any molecule with an amino acid sequence derived at least in part from that of thrombin, whether synthesized in vivo or in vitro. Accordingly, a thrombin derivative, as referred to herein, designates a polypeptide molecule which comprises fewer amino acids than thrombin.

A physiologically functional equivalent of a thrombin derivative encompasses molecules which differ from thrombin derivatives in particulars which do not affect the function of the thrombin receptor binding domain or the serine esterase conserved amino acid sequence. Such particulars may include, but are not limited to, conservative amino acid substitutions and modifications, for example, amidation of the carboxyl terminus, acetylation of the amino terminus, conjugation of the polypeptide to a physiologically inert carrier molecule, or sequence alterations in accordance with the serine esterase conserved sequences.

A thrombin receptor binding domain is defined as a polypeptide sequence which directly binds to the thrombin receptor and/or competitively inhibits binding between high-affinity thrombin receptors and alpha-thrombin.

A domain having a serine esterase conserved sequence comprises a polypeptide sequence containing at least 4–12 of the N-terminal amino acids of the dodecapeptide previously shown to be highly conserved among serine proteases (Asp-X_1-Cys-X_2-Gly-Asp-Ser-Gly-Gly-Pro-X_3-Val);
wherein X_1 is either Ala or Ser; X_2 is either Glu or Gln; and X_3 is either Phe, Met, Leu, His, or Val).

A stimulatory polypeptide is defined as a polypeptide derivative of thrombin, or a physiologically functional equivalent thereof, having the ability to both bind to and stimulate the thrombin receptor. Therefore, the stimulatory peptides will include both a thrombin receptor binding domain and a domain with a serine esterase conserved amino acid sequence.

An inhibitory polypeptide is defined as a polypeptide derivative of thrombin, or a physiologically functional equivalent thereof, having a thrombin receptor binding domain but without a serine esterase conserved amino acid sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A–1D. Computer assisted analysis of the hydropathy, solubility, and predicted secondary structure for residues 489 to 548 of human prothrombin. FIG. 1A, hydropathy profile; FIG. 1B, solubility profile; FIG. 1C, predicted tendency for flexible turn; FIG. 1D, predicted tendency for alpha-helix and beta-sheet structure.

FIGS. 2A–2C. Three-dimensional representation of X-ray crystallographic data of trypsin with the following PROTEUS computer-assisted substitutions of thrombin-specific residues: Gly_{187} Lys; Lys_{188} Arg; Ser_{190} Ala; Gln_{192} Glu; and Val_{199} Phe is shown in FIGS. 2A and 2D. FIGS. 2B and 2C show only the three active site residues (His_{57}, Asp_{102}, Ser_{195}) and residues 183 to 200 of trypsin that are located in the homologous region as thrombin's residues 510 to 530. These peptides are oriented in the same position as in the rotated model in FIG. 2A and 2D.

FIG. 3. Inhibition of [^{125}I]-alpha-thrombin binding to mouse embryo (ME) cells by synthetic peptide p508-530. Specific binding of 0.3 nM [^{125}I]-alpha-thrombin to ME cells in the presence of the indicated concentration of peptide was measured as described in the description of the preferred embodiments.

FIG. 4 Effect of p508-530 on [^{3}H]-thymidine incorporation alone or in combination with low concentrations of alpha-thrombin. Quiescent serum-free cultures of ME (FIG. 4A) or NIL (a hamster fibroblast cell line; FIG. 4B) were treated with the indicted concentrations of p508-530 alone (O) or in combination with concentrations of alpha-thrombin which gave approximately one third of the maximal response; 2 nM for ME cells (FIG. 4A) and 4 nM for NIL cells (FIG. 4B). [^{3}H]-thymidine incorporation was determined after 24 hours as described in the description of the preferred embodiments.

FIG. 5 Effect of p508-530 on [^{3}H]-thymidine incorporation in combination with PMA. Quiescent cultures of NIL cells were incubated with p508-530 alone (O) or in combination with 25 ng/ml PMA. [^{3}H]-thymidine incorporation was determined as described in the description of the preferred embodiments.

FIG. 6. Comparison between effects of peptides on thrombin-stimulated thymidine incorporation. Quiescent cultures of NIL cells were incubated with increasing concentrations of p508-530, p519-530, or p517-520 in the presence of 1 nM alpha-thrombin (a marginally mitogenic concentration). Data are expressed-for each concentration as a percentage of the effect of alpha-thrombin alone.

FIG. 7. Effect of p517-520 on thrombin stimulation of [^{3}H]-thymidine incorporation. Quiescent cultures of ME cells were incubated with increasing concentrations of alpha-thrombin alone, or in combination with 625 nM p517-520. [^{3}H]-thymidine incorporation was determined as described in the description of the preferred embodiments.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Thrombin, a molecule once considered important only in the context of blood coagulation, is now shown to mediate a number of potent biological effects not directly related to coagulation. Many of these effects are due, at least in part, to signals generated by the interaction between thrombin or thrombin-like molecules and the high-affinity thrombin receptors present on the surface of many cells.

Studies performed in connection with the present invention suggested that selective regulation of thrombin-mediated events might be achieved through the formulation and synthesis of polypeptides specifically designed to either mimic or inhibit such events. Development of small protease inhibitor resistant polypeptides capable of performing these functions was particularly desirable in view of the susceptibility of thrombin to proteolytic enzyme inhibitors, such as antithrombin.

A number of peptides based on the sequence of human prothrombin were synthesized and tested for their ability to bind to the receptor and to generate proliferative signals. The choice of peptides focused on the amino acid sequence of the region of thrombin around its active site serine. This region contains a domain (represented by residues 517-520 of human prothrombin) with a sequence homologous to the tripeptide cell binding domain of fibronectin, [Arg-Gly-Asp]. This tripeptide sequence is common to a number of proteins that may interact with cells (reviewed by Rouslahti and Peirschbacher, *Cell*, 44:517–518 (1985)). Moreover, it has been shown that a peptide representing 517-520 of human prothrombin (p517 -520) and peptides representing 516-522 and 510-526 of human prothrombin (p516-522 and p510-526, respectively) are able to promote fibroblast attachment comparable to that induced by fibronectin-specific peptides.

The selected region also possesses a domain (represented by residues 519-530 of human prothrombin) with a high degree of homology to a number of serine esterases.

The present inventors have discovered that a synthetic peptide containing both fibronectin- and serine protease-homologous domains (residues 508 to 530 of human prothrombin) binds to thrombin receptors with high-affinity and substitutes for DIP-alpha-thrombin as an initiator of receptor occupancy-related mitogenic signals. In contrast, a synthetic peptide containing only the fibronectin-homologous domain (p517-520) binds to the thrombin receptor without inducing mitogenesis. An intermediate peptide (p519-530) retains the ability to mediate mitogenesis but to a much lesser degree than p508-530.

EXAMPLE 1

Selection of Domains of Human Alpha-Thrombin Involved in Binding of Thrombin to Its High Affinity Receptor

To help select peptide sequences that might be involved in receptor binding, computer analysis was used to predict the overall hydropathy, solubility, and secondary structural features for the 60 amino acid residues around the active site serine of alpha-thrombin based on the sequence of human prothrombin (Degen et al., *Biochem.*, 22:2087–2097 (1983)). As shown in FIGS. 1A and 1B, this region appears to be highly hydrophilic and soluble, especially near the region that is homologous to fibronectin's cell attachment domain, residues 517 to 520. Analysis of secondary structural features indicated that the region of thrombin from residues 511 to 526 has a strong tendency for being a flexible turn region with very little tendency towards either alpha-helical or beta-sheet structures (FIGS. 1C and 1D). Taken together, the various computer-assisted analyses strongly suggest that this region of thrombin should be externally accessible and, therefore, available for interaction with the thrombin cell surface receptor. Moreover, the region of thrombin homologous to the cell attachment domain of fibronectin is located at or very near the middle of this hydrophilic flexible turn of thrombin.

Using the three dimensional x-ray crystallographic data for trypsin (Marquart et al., *Acta. Crystallogr.*, 39:480 (1983)), and making appropriate amino acid substitutions to reflect the thrombin sequence around the active-site serine portion of trypsin, computer graphic analysis predicted that residues 510 to 530 of thrombin are located along the edge of the pocket that leads to the active site cleft (FIG. 2). In agreement with the predictions of secondary structure discussed above, amino acid residues 517 to 520 of thrombin are located at the outer most corner of this region of the proposed trypsin/thrombin structure. Thus, it appeared reasonable that this region of thrombin could be involved in binding to its receptor.

EXAMPLE 2

Synthesis of Peptides

Peptides were synthesized by the solid-phase method (Erickson and Merrifield, *The Proteins*, 2:255–257, (1976)) using automatic instrumentation (Applied Biosystems Peptide Synthesizer Model 430A) and purified by HPLC (Beckman) on a C-18 column eluted with a linear acetonitrile gradient containing 0.5% (v/v) TFA (trifluoroacetic acid).

EXAMPLE 3

Demonstration that the Thrombin Derivatives Selectively Bind to the High-Affinity Thrombin Receptor

This example demonstrates that the peptides of the present invention are able to selectively bind to the high-affinity thrombin receptors present on the surfaces of many cell types. In the present embodiment, this activity was demonstrated by showing that the peptides of the present invention competitively inhibited binding of [^{125}I]-alpha-thrombin to thrombin receptors present on two strains of cultured fibroblasts. Accordingly, the specific techniques described below represent the best mode for demonstrating this activity known to the inventors at the present time.

a. Culture of Fibroblasts Having High-Affinity Thrombin Receptors

As stated above, fibroblasts derived from two sources were used to demonstrate binding of the peptides of the present invention to high-affinity thrombin receptors. These cell lines were prepared as follows:

Primary cultures of fibroblasts were prepared from 9- to 13-day old embryos of NIH-swiss outbred mice as described by Carney and Cunningham, *Cell*, 15:1341–1349, (1978). NIL cells, an established strain of hamster fibroblasts, were maintained as stock cultures and subcultured every four days. All cells were grown in Dulbecco-Vogt modified Eagle's (DV) medium supplemented with 10% (v/v) bovine calf serum (CS), in a humidified atmosphere of 5% CO_2 in air at 37° C.

Quiescent cultures were prepared by subculturing stock cells from 100 mm dishes, using 0.05% (w/v) trypsin and 0.02% EDTA (w/v) in phosphate-buffered saline (PBS) and plating them in 24-well culture plates in DV medium supplemented with 10% (v/v) CS at 6×10^4 cells/cm^2. After allowing the cells to attach overnight, the medium was removed and the cells were rinsed with DV medium containing no serum. The cells were incubated in this serum-free medium for 48 hours before the indicated experiments. This procedure has been shown to provide nonproliferating populations of mouse and NIL cells that are 90–95% arrested at the G_1/G_0 cell cycle interface.

b. Assay for Measurement of Specific Binding of Thrombin and Thrombin Derivatives to the Cell Surface Thrombin Receptor

As stated above, in the present embodiment, thrombin receptor specific binding activity of the thrombin derivatives was measured as a function of their ability to competitively inhibit binding between native [^{125}I]-thrombin and the thrombin receptor. Specific techniques whereby the competitive binding studies were performed are set out below.

Human alpha-thrombin was iodinated in the presence of benzamidine (an active-site competitive inhibitor), lactoperoxidase, and Na[^{125}I]. After gel filtration and dialysis, the [^{125}I]-alpha-thrombin had a specific activity of 1 to 3×10^{-7} CPM/ug and co-migrated with unlabeled alpha-thrombin as a single band on sodium dodecyl sulfate (SDS) polyacrylamide gels. These iodinated preparations retained approximately 80% of their fibrinogen clotting activity.

The ability of the synthetic peptides to compete for specific [^{125}I]-alpha-thrombin binding to fibroblasts was measured on nonproliferating, mitogenically responsive cultures in 24 well plates (Falcon) at a cell density of approximately 5×10^4 cells/cm^2 as previously described (Carney and Cunningham, *Cell*, 15:1341–1349 (1978)). The medium on the cells was changed to binding medium (serum-free DV medium containing 0.5% (w/v) bovine serum albumin buffered with 15 mM HEPES at pH 7.0). The cells were equilibrated at 23° C. for 30 minutes, and the medium was changed to binding medium containing [I^{125}I]-alpha-thrombin (10 ng/ml) with the indicated concentrations of the peptides. After 2 hours at 23° C., the assay was terminated by quickly rinsing the cells four times with ice-cold PBS. The cells were dissolved in 1 ml of 0.5N NaOH and the total radioactivity was measured using a Beckman gamma counter. Nonspecific binding was measured as the radioactivity bound to cultures after incubation in binding medium containing a 100-fold excess of unlabeled alpha-thrombin. Specific binding was calculated by subtracting nonspecific binding from total radioactivity bound to cultures.

c. Thrombin Binding Activity of Selected Thrombin Derivatives

In order to demonstrate the thrombin receptor binding activity of the polypeptides of the present invention, the peptides synthesized as described in Example 1 were tested for thrombin receptor activity using the assay system described immediately above.

More specifically, in order to demonstrate that p508-530 bound to thrombin receptors, confluent cultures of ME cells were incubated with 0.3 nM [^{125}I]-alpha-thrombin and concentrations of p508-530 ranging from 8 to 4000 nM for 90 minutes at 23° C. As shown in FIG. 3, p508-530 competed for 30% to 70% of the specific binding of [^{125}I]-alpha-thrombin to ME cells. Scatchard analysis of the direct binding of [^{125}I]-labeled p508-530 indicated a K_D of approximately 6×10^{-8}M (data not shown). In addition, the specific binding of [^{125}I]-p508-530 to ME cells could be displaced by both excess p508-530 or excess human alpha-thrombin. Thus, it appears that the competition of p508-530 for [^{125}I]-alpha-thrombin binding represents the binding of p508-530 to the same sites as alpha-thrombin, but with an affinity approximately one order of magnitude lower.

Furthermore, in order to show that the binding and mitogenic activity of p508-530 was specific, two synthetic peptides with physical properties similar to p508-530 but no sequence homology to human alpha-thrombin were tested for their binding properties. Both of these peptides [one with 12 amino acids (33% hydrophobic residues and a net charge of –3) and a second with 18 amino acids (39% hydrophobic

residues and a net charge of 0)] inhibited binding of [^{125}I] -alpha-thrombin less than 5% at concentrations up to 5uM.

To further identify regions of thrombin involved in high-affinity binding and generation of mitogenic signals, two peptides representing specific domains within p508-530 were tested. The first peptide represented residues 519 to 530 of the B-chain region of human prothrombin, a region of thrombin that is highly conserved among serine proteases. The second peptide represented residues 517 to 520 of prothrombin, a region of thrombin homologous to the fibronectin cell binding domain.

Both of these peptides were able to compete for 30% to 50% of the binding of [^{125}I]-alpha-thrombin to ME cells, but both required higher concentrations than was required with the initial peptide p508-530 (Table 1). For example, 30% inhibition of [^{125}I]-alpha-thrombin binding required 33- to 50-fold higher concentrations of p519-530 and p517-520 than p508-530, respectively. Thus, both of these peptides appear to interact with thrombin receptors, but at a lower affinity than p508-530. Because p517-520 is homologous to the fibronectin cell binding domain, a peptide having the sequence Arg-Gly-Ala-Ser (the sequence of the fibronectin specific peptide) was also tested for its ability to compete for [^{125}I]-alpha-thrombin binding. At a concentration of 1.3 uM, this peptide did not compete with [^{125}I]-alpha-thrombin for binding. Thus, the receptor for alpha-thrombin is not the same membrane protein that specifically interacts with fibronectin and causes the apparent growth promoting action of fibronectin. In addition, these results demonstrate the requirement for alanine within the thrombin receptor binding domain, since substitution of alanine with serine eliminated the ability of the synthetic peptide to compete for alpha-thrombin binding.

TABLE 1

Comparison of Peptide Competition for [^{125}I]-Alpha-Thrombin Binding to ME Cells.

Peptide	Amino Acid Sequence	Concentration Required for 30% Inhibition	Maximal % Inhibition (and Conc.)
p508-530	AGYKPDEG--KRGDACE--GDSGGPFV	6 nM	78% (40 nM)
p519-530	DACEGD--SGGPFV	200 nM	51% (800 nM)
p517-520	RGDA	300 nM	50% (2.7 uM)

Various concentrations of peptides and [^{125}I]-alpha-thrombin (1 nM) were incubated with quiescent ME cells for minutes at 23° C. Specific binding of [^{125}I]-alpha-thrombin was defined as described in Example 3.

EXAMPLE 4

Stimulation of DNA Synthesis by Selected Thrombin Derivatives

This example demonstrates that binding between stimulatory (agonistic) polypeptides and thrombin receptors generates a receptor occupancy signal which induces DNA synthesis and cell division. In the present embodiment, DNA synthesis and cell proliferation was measured as a function of [^{3}H] thymidine uptake by cultured fibroblasts exposed to selected polypeptides in the presence of non-mitogenic concentrations of alpha-thrombin or PMA. Although the in vitro techniques described below represent the best mode for demonstrating the stimulatory activity of the selected polypeptides, those skilled in the art will appreciate that the principles demonstrated in the in vitro system described immediately below are also applicable in vivo.

a. Techniques for Measuring DNA Synthesis

The effects of the synthetic peptides on DNA synthesis were determined by measuring the incorporation of methyl-[^{3}H]-thymidine (TdR, ICN Pharmaceuticals, Irvine, Calif.) during a 2 hour incubation generally from 22 hours after addition of peptides and/or thrombin (Stiernberg et al., *J. Cell Physiol.*, 120:209–285 (1984)). After incubation, the cells were extracted and rinsed with ice-cold 10% (w/v) trichloroacetic acid (TCA). The acid precipitable material was dissolved overnight in 0.5 ml 0.5 N KOH at 23° C. HCl (1 N), 0.25 ml, was added and the solution was counted in 10 ml of RediSolv-HPb (Beckman Instruments, Houston, Tex.) scintillation fluid.

b. Mitogenic Activity of Selected Thrombin Derivatives

Each of the thrombin derivatives synthesized was tested for mitogenic activity as were the two non-thrombin peptides described in Example 3(c). The results of these experiments are described below.

The present inventors first tested the ability of p508-530 to stimulate DNA synthesis in non-proliferating cultures of ME or NIL cells. As shown in FIG. 4, p508-530, by itself, was not sufficient to stimulate [^{3}H]-thymidine incorporation into DNA. However, in combination with 2 nM alpha-thrombin, 0.1 uM p508-530 stimulated a 6- or greater than 2-fold increase in incorporation of [^{3}H]-thymidine into DNA in ME cells when compared to parallel cultures left untreated or treated with alpha-thrombin alone, respectively (FIG. 4A). A similar mitogenic stimulation was also observed in NIL hamster cells, although it required a slightly higher concentration of thrombin and peptides (FIG. 4B). The responses in both cell types were equivalent to the mitogenic response stimulated by a maximally effective concentration of alpha-thrombin (10 nM). It is noteworthy that for ME cells, stimulation by p508-530 was observed between 12.5 nM and 100 nM (FIG. 4A), concentrations that correspond closely with those required to inhibit [^{125}I]-alpha-thrombin binding to ME cells (FIG. 3). With NIL cells, a similar correlation was observed between the mitogenic concentrations of p508-530 and the concentrations required to inhibit thrombin binding, although at higher levels than required with ME cells.

Although these results suggest that p508-530 generates mitogenic signals through its interaction with high-affinity thrombin receptors, it was possible that the peptide merely increased the effective concentration of alpha-thrombin. Recently, phorbol myristate acetate (PMA) has been shown to mimic the effects of gamma-thrombin and stimulate DNA synthesis and cell proliferation in combination with DIP-alpha-thrombin or with monoclonal antibodies to the thrombin receptor. It was predicted, therefore, that if p508-530 was generating a receptor occupancy-related signal, its addition to cells in combination with PMA should stimulate mitogenesis. As shown in FIG. 5, in the presence of 25 ng/ml PMA (which is a non-mitogenic amount), p508-530 stimulated a 3.5-fold increase in DNA synthesis over controls. This stimulation occurred at approximately the same concentration of peptide as that required to stimulate DNA synthesis in the presence of low concentrations of alpha-thrombin. Since active thrombin was not present in these experiments, it would appear that p508-530 itself generates a mitogenic signal that mimics the effect of DIP- or alpha-thrombin binding to high-affinity thrombin receptors.

In order to ensure that the stimulation of DNA synthesis by p508-530 was mediated by virtue of its ability to interact

with the high-affinity thrombin receptor, the synthetic, non-thrombin, non-receptor binding polypeptides described in Example 3(c) were tested for mitogenic activity. Neither of these peptides generated a mitogenic response in the presence of 1 nM alpha-thrombin. Thus, neither the binding activity nor the mitogenic activity of p508-530 is due to non-specific interaction of the polypeptide with the cells.

The inventors then tested the mitogenic activity of the smaller thrombin derivatives, p519-530 and p517-520. As indicated in Example 3(c) above, both of these peptides bind to the high-affinity thrombin receptor. In these experiments, increasing concentrations of p519-530 and p517-520 were added to quiescent NIL cells in the presence of 2 and 4 nM alpha-thrombin. As shown in FIG. 6, p519 enhanced DNA synthesis over a range of concentrations while p517-520 did not. In fact, p517-520 actually inhibited DNA synthesis.

EXAMPLE 5

Inhibition of Thrombin-Receptor Mediated Mitogenesis by p517-520

The observation that p517-520 inhibits alpha-thrombin stimulated mitogenesis was somewhat startling in view of previous studies demonstrating that mitogenic and transmembrane signaling effects of thrombin were not inhibited by DIP-alpha-thrombin, a thrombin derivative which competes for active alpha-thrombin binding. Thus, the inventors realized that p517-520, which is able to compete with native alpha-thrombin for binding to high-affinity cell surface thrombin receptors, but is unable to generate the mitogenic receptor occupancy signal, has properties not previously known in the art.

In order to explain the mechanism by which p517-520 was able to inhibit thrombin-mediated mitogenesis, the inventors measured the ability of increasing concentrations of alpha-thrombin to stimulate DNA synthesis in cultures to which a constant concentration (625 nM) of p517-520 had been added (FIG. 7). These experiments showed that p517-520 significantly shifted the doseresponse curve of the cells to alpha-thrombin. For example, at two concentrations of alpha-thrombin, 0.8 and 13.0 nM, DNA synthesis was inhibited by approximately 75% and 35%, respectively. Thus, the inhibition of alpha-thrombin stimulation by p517-520 appears to require a 500–1000 fold molar excess of the peptide. This finding is consistent with the observation that p517-520 has a lower competitive binding affinity for thrombin receptors on ME cells than does p508-530.

The identification of p517-520 as the high-affinity binding domain of thrombin has several implications as to the mechanism of thrombin mitogenesis. Previous studies have demonstrated proteolytic cleavage and disappearance of a molecule on the surface of chick embryo cells treated with thrombin. Cross-linking studies with active or inactive thrombin have also identified two differently sized receptor molecules or substrates. The present results show that the high-affinity binding domain of thrombin is very close to the active-site cleft; thus, it should be possible for thrombin to cleave its receptor. Preliminary data from affinity purification of the thrombin receptor supports the hypothesis that the receptor itself is proteolytically cleaved by active thrombin. It is possible that thrombin receptor occupancy may stimulate an alteration in receptor conformation necessary for the cleavage event. The present results suggest that peptides p508-530, p519-530 or alpha-thrombin itself are capable of binding to the thrombin receptor in a manner which induces such confirmational changes. In contrast, p517-520 appears to be capable only of binding to the receptor. Thus, p517-520 selectively inhibits thrombin receptor-mediated events by virtue of its ability to selectively interact with thrombin receptors in a manner which provides the cell with a null signal.

EXAMPLE 6

Use of Stimulatory Peptides to Potentiate Cell Growth In Vitro

A number of experimental and diagnostic procedures require cells grown in vitro. Because the stimulatory peptides enhance proliferation of fibroblastic cells bearing high-affinity thrombin receptors, the incorporation of such stimulatory molecules into the culture medium will provide an effective means of potentiating cell growth. In addition, because thrombin stimulates proliferation of other cells, including endothelial cells, these peptides may be effective in promoting growth of a number of types of cells. Use of the synthetic polypeptides as growth supplements has a number of advantages. It is much less expensive to synthesize the polypeptides than its is to purify naturally occurring thrombin. Furthermore, unlike naturally occurring thrombin, the polypeptides are relatively resistant to inhibition by serum protease inhibitors.

Numerous methods for preparing cells for culture are known to those skilled in the art. One such method, described by Carney et al. (*J. Cell. Physiol.*, 95:13–22, 1978, incorporated herein by reference), is believed to be particularly well suited to the practice of this aspect of the invention.

As will be appreciated by those of skill in the art, the stimulatory polypeptides of the present invention may be employed together with any suitable cell culture medium to achieve the advantages of their cell-stimulation effects. For example, the present inventors have found a mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F12 medium to be a particularly appropriate base medium. To practice the invention, one adds 0.1 ug/ml–10 ug/ml of the stimulatory peptide Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val to the culture medium. The cells are then incubated in an appropriate humidified atmosphere, for example, one containing 5% CO_2 in air at 37° C. At regular intervals (3 or 4 days), the spent medium is removed from the cell culture and replaced with fresh medium formulated as described above.

EXAMPLE 7

Treatment Protocols

Due to precautions necessarily attendant to development of every new pharmaceutical, the polypeptides of the present invention have not yet been tested in a clinical setting in human subjects. However, the in vitro activity of these polypeptides in selectively promoting or inhibiting thrombin-mediated mitogenesis is believed to demonstrate the utility of the present invention in this regard. The following prophetic embodiments represent the best mode contemplated by the present inventors of carrying out the practice of the invention in various clinical settings.

a. Wound Healing

It is believed that the stimulatory polypeptides will prove to be useful in numerous clinical situations where it is desirable to potentiate wound healing. In particular, these

include treatment of burn patients, those involved in severe accidents, those subjected to a variety of surgical procedures and those with poor wound healing responses, such as the aged and diabetic. Although the best mode of administering the polypeptides will depend on the particular clinical situation, it is believed that its administration in the form of an aerosol spray will prove to be particularly advantageous in a number of such settings. Methods for incorporating therapeutic agents into aerosol sprays are well known in the art. Therefore, it is considered that formulation and use of these stimulatory polypeptides in such aerosol sprays is well within the skill of the art in light of the present disclosure.

The stimulatory polypeptide may also be applied to the wound in the form of a salve or lotion. Alternatively, they may be incorporated into the material used to dress the wound. Techniques for incorporation of therapeutic agents compositions into salves, lotions and wound dressings are also well known in the art and within the skill of the art in light of the present specification.

It is believed that an effective dose of the polypeptide is approximately between 0.5 uM–50 uM. However, exact dosages would, of course, be determined empirically by experimental methods well known to those skilled in the pharmaceutical arts.

b. Use of the Inhibitory Polypeptides

1. Inhibition of Scar Formation and Formation of Tissue Adhesions

It is further believed that the inhibitory polypeptides will prove useful in a number of situations, for example, where inhibition of fibroblast proliferation is desirable. These include prevention of scar formation and tissue adhesions.

One manner in which the invention may be practiced is by incorporating the inhibitory polypeptide Arg-Gly-Asp-Ala into any vehicle suitable for application to a wound, surgical incision or surface of a body organ. These vehicles include aerosol sprays, salves and lotions appropriate for direct application to tissues as well as solutions appropriate for intravenous or subcutaneous injections. Methods for incorporating therapeutic agents into pharmaceutical vehicles such as those described above is believed to be well within the skill of the art, as are methods for applying the resultant compositions.

It is proposed that an effective dose of the polypeptide is 1 ng/cm^2–10 ug/cm^2 if the compound is applied topically. If injected, an effective dose is that dose sufficient to obtain a concentration of the polypeptides of from 0.1 uM to 10 uM, at the site where needed. However, exact doses, of course, should be determined by accepted pharmaceutical methods known to those skilled in the pharmaceutical arts.

2. Tumor Therapy

It is believed that the inhibitory polypeptides will further prove to be useful in the treatment of various tumors, particularly in preventing metastasis and angiogenesis. It is anticipated that the inhibitory polypeptides could best be administered by intravenous administration.

The inhibitory polypeptides could be given daily by continuous infusion or on alternative days, with more traditional chemotherapy being given on the intervening day. While exact doses of the inhibitory peptides would have to be determined empirically by methods known to those skilled in the art, it is estimated that an effective dose would be that amount sufficient to achieve a concentration of 0.1 uM to 10uM at the site where needed. Of course, as with a new pharmaceutical agent of any type, clinical trials would be needed to establish levels at which unacceptable toxicity would be reached.

The present invention has been disclosed in terms of specific embodiments believed by the inventor to be the best mode for carrying out the invention. However, in light of the disclosure hereby provided, those of skill in the various arts will recognize that modifications can be made without departing from the intended scope of the invention. For example, any of these peptides may be administered by a number of methods known in the art. Furthermore, future studies are expected to result in production of thrombin derivatives with increased stimulatory or inhibitory activity. These and all other modifications and embodiments are intended to be within the scope of the claims.

What is claimed is:

1. A method for promoting wound healing comprising

applying to a wound a therapeutically effective amount of a purified thrombin derivative peptide of 23 amino acids in length wherein said peptide includes a

a thrombin receptor binding domain having the sequence Arg-Gly-Asp-Ala;

a thrombin receptor binding domain having the sequence of Arg-Gly-Asp-Ala;

a serine esterase conserved sequence; and

wherein the Asp-Ala of the thrombin receptor binding domain comprise the first two amino acids of the serine esterase conserved sequence.

2. The method according to claim **1** further comprising applying to the wound a therapeutically effective amount of a compound selected from the group consisting of alpha-thrombin or gamma thrombin in order to promote wound healing.

3. The method of claim **1** wherein the serine esterase conserved sequence comprises Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

4. The method of claim **1** wherein the thrombin derivative peptide has the amino acid sequence: Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

5. The method of claim **1** wherein the thrombin derivative peptide consists of the amino acid sequence Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

6. The method of claim **1** wherein the serine esterase conserved sequence is Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,500,412

DATED : March 19, 1996

INVENTOR(S) : Darrell H. Carney and Kevin C. Glenn

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In claim 1, column 16, line 31-32, delete "a thrombin receptor binding domain having the sequence of Arg-Gly-Asp-Ala;"

Signed and Sealed this

Twenty-fifth Day of June, 1996

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

[redacted]

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,500,412

DATED : March 19, 1996

INVENTOR(S) : Darrell H. Carney and Kevin C. Glenn

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, please insert the following item:

"[73] Assignee: Board of Regents, The University of Texas System, Austin, Texas."

Signed and Sealed this

Third Day of June, 1997

Attest:

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

[redacted]

US005352664A

United States Patent [19]

Carney et al.

[11] Patent Number: 5,352,664

[45] Date of Patent: Oct. 4, 1994

[54] **THROMBIN DERIVED POLYPEPTIDES; COMPOSITIONS AND METHODS FOR USE**

[75] Inventors: **Darrell H. Carney**, Galveston, Tex.; **Kevin C. Glenn**, St. Louis, Mo.

[73] Assignees: **Board of Regents, The University of Texas System**, Austin, Tex.; **Monsanto Company**, St. Louis, Mo.

[21] Appl. No.: **925,201**

[22] Filed: **Oct. 31, 1986**

[51] **Int. Cl.**[5] **A61K 37/02**; A61K 37/547; C07K 7/10; C12N 9/74

[52] **U.S. Cl.** **514/13**; 530/326; 435/214; 424/94.64

[58] **Field of Search** 530/330, 327, 326; 514/2, 13, 14, 18; 435/214; 424/94.64

[56] **References Cited**

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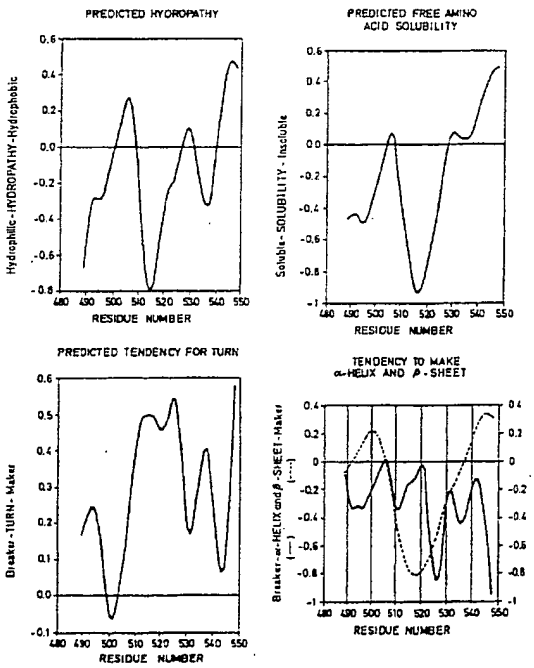
Primary Examiner—George C. Elliott
Attorney, Agent, or Firm—Arnold, White & Durkee

[57] **ABSTRACT**

Thrombin is now known to mediate a number of potent biological effects on cells bearing high-affinity thrombin receptors. These effects depend, at least in part, upon receptor occupancy signals generated by thrombin's interaction with the high affinity thrombin receptor. The present inventors have formulated synthetic thrombin derivatives capable of selectively stimulating or inhibiting thrombin receptor occupancy signals. The stimulatory thrombin derivatives to bind to cell surface thrombin receptors and stimulate DNA synthesis in cells treated with non-mitogenic concentrations of alpha-thrombin or phorbol myristate acetate. Thus, these peptides, which have both a thrombin receptor binding domain and a segment of amino acids with a sequence common to a number of serine proteases, appear to generate receptor-occupancy dependent mitogenic signals. The inhibitory derivatives, which have no serine esterase conserved amino acid sequences bind to thrombin receptors without generating receptor-occupancy dependent mitogenic signals. This invention describes the peptides and methods for using them to promote cell growth and wound healing or to inhibit scar formation, tissue adhesions, and tumor metastasis and angiogenesis.

6 Claims, 6 Drawing Sheets



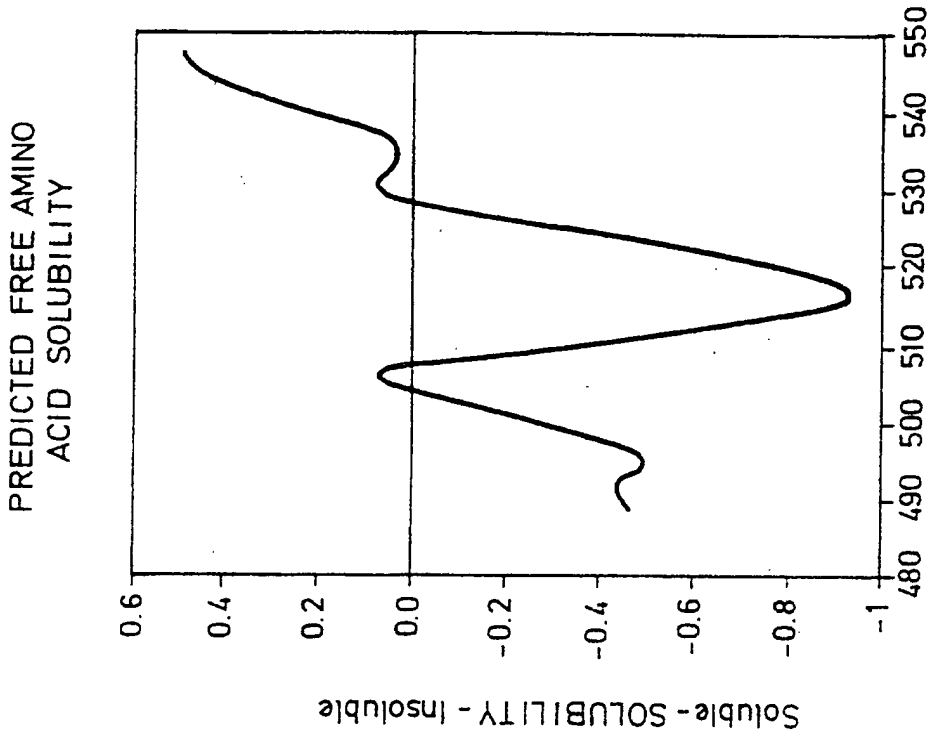

PREDICTED FREE AMINO ACID SOLUBILITY
Soluble - SOLUBILITY - Insoluble
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1
480 490 500 510 520 530 540 550
RESIDUE NUMBER

Fig-1B

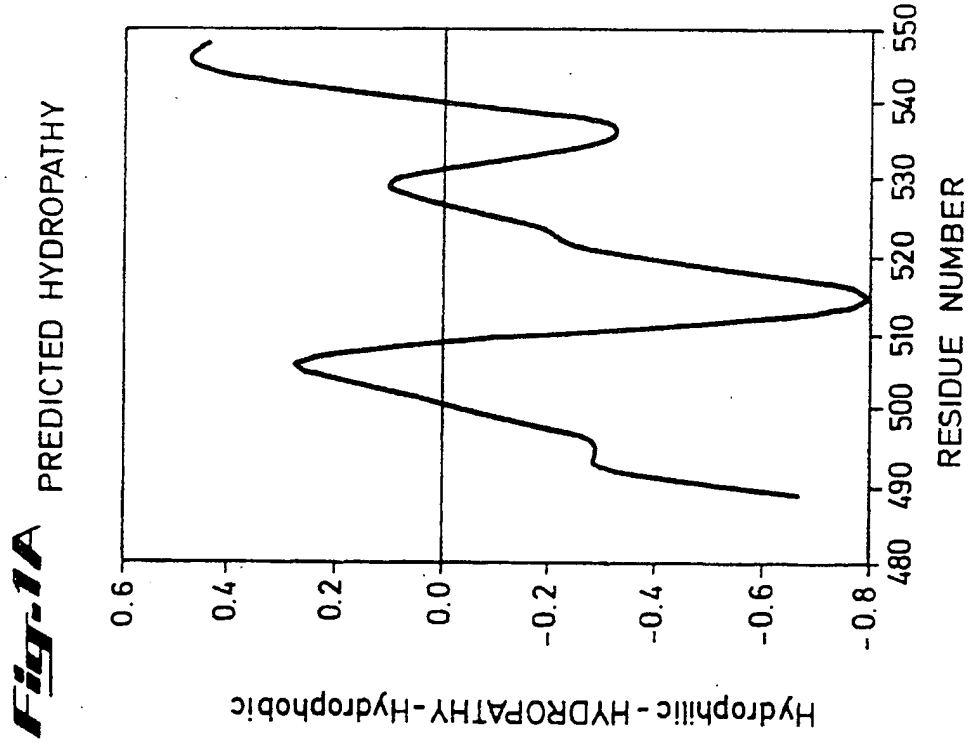

PREDICTED HYDROPATHY
Hydrophilic - HYDROPATHY - Hydrophobic
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
480 490 500 510 520 530 540 550
RESIDUE NUMBER

Fig-1A

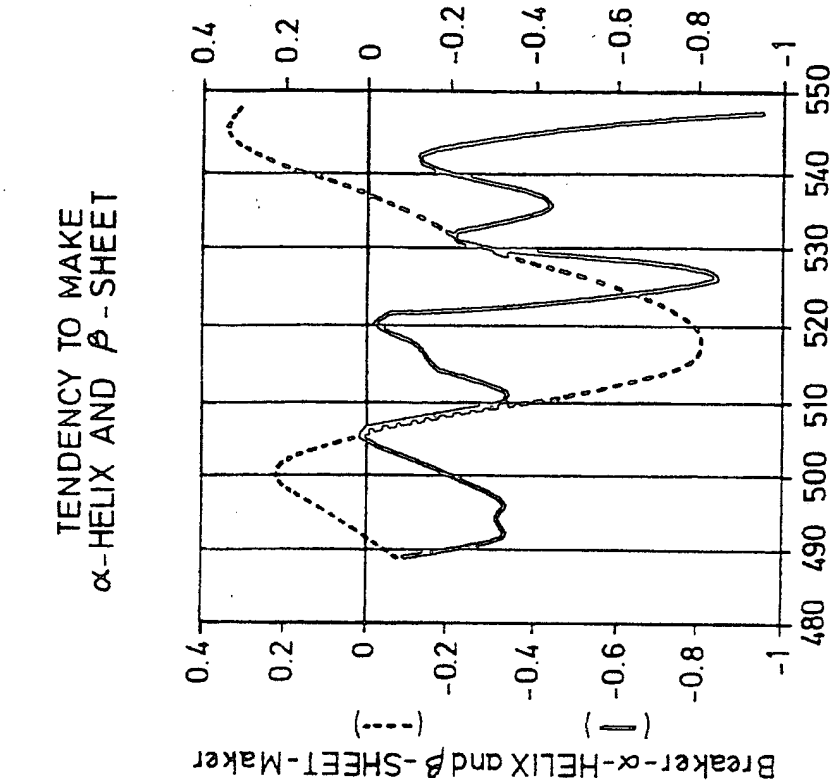
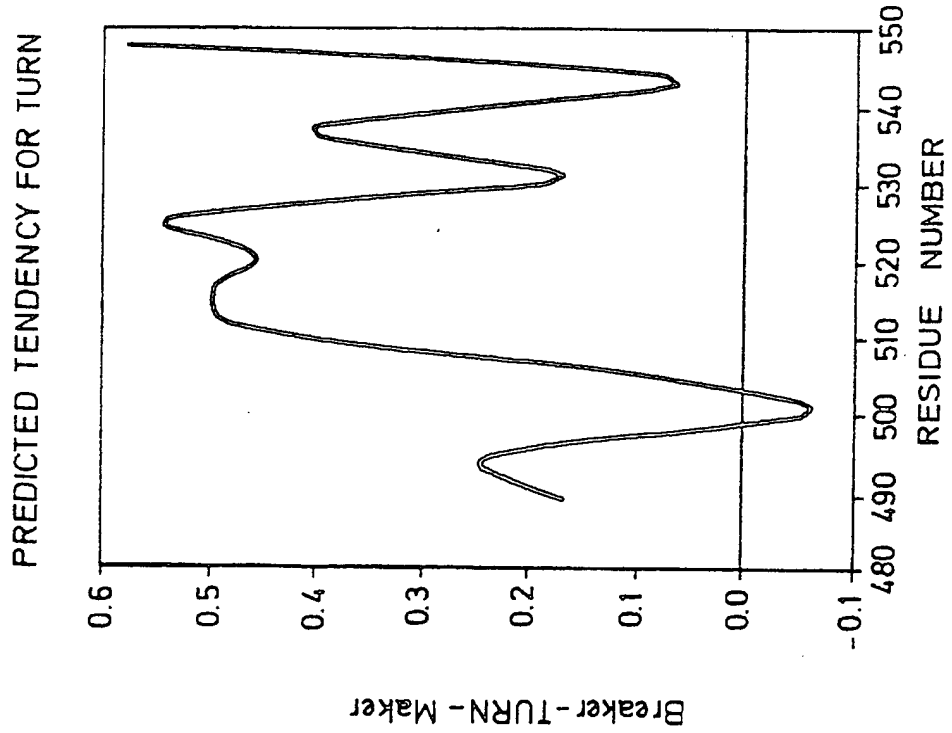

TENDENCY TO MAKE
α-HELIX AND β-SHEET
RESIDUE NUMBER
480 490 500 510 520 530 540 550
0.4 0.2 0 -0.2 -0.4 -0.6 -0.8 -1
Breaker-α-HELIX and β-SHEET-Maker
(----) (—)

Fig-1D


PREDICTED TENDENCY FOR TURN
RESIDUE NUMBER
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Breaker-TURN-Maker

Fig-1C

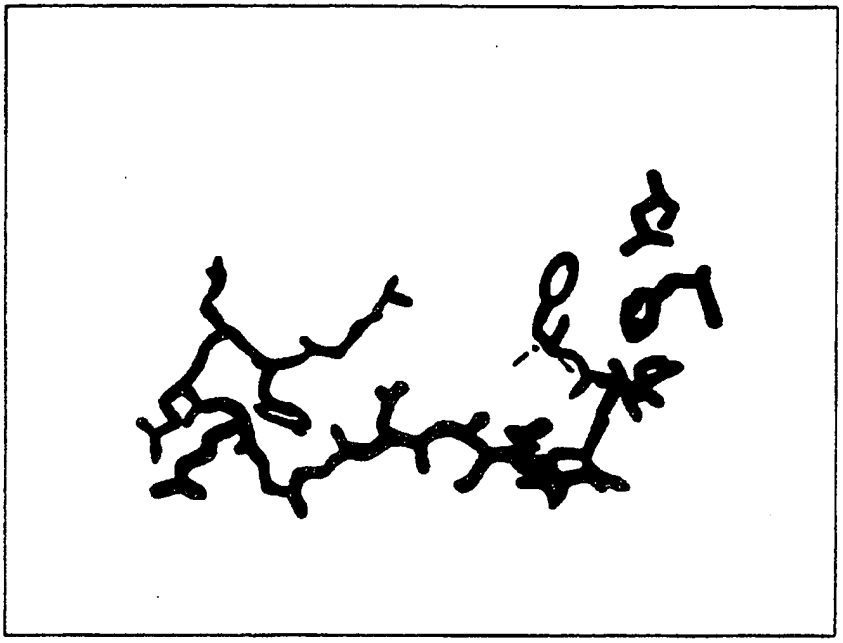


Fig.2B

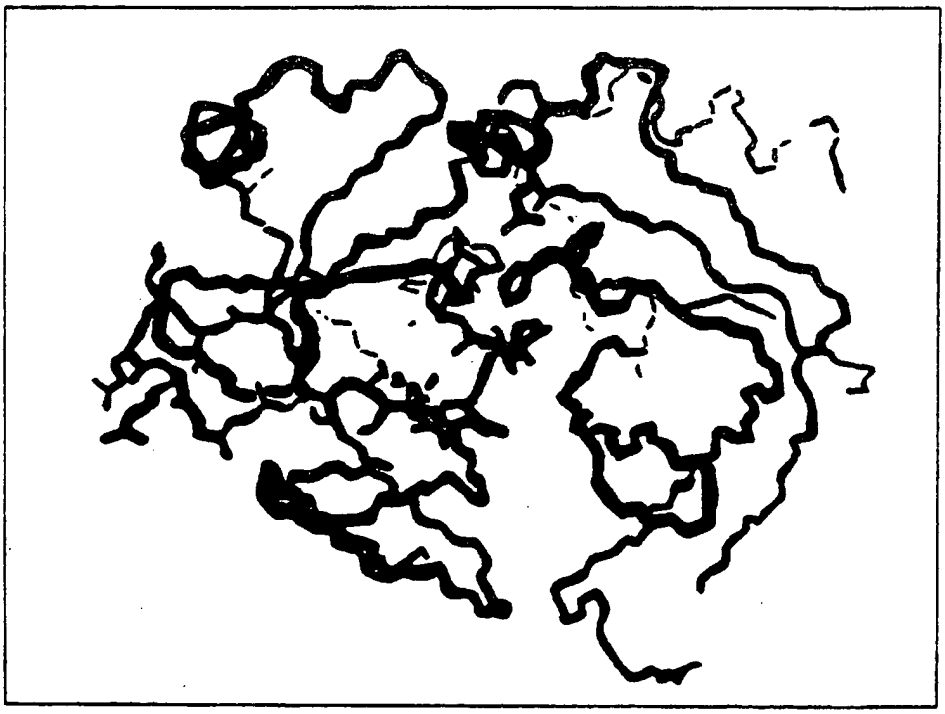


Fig.2A

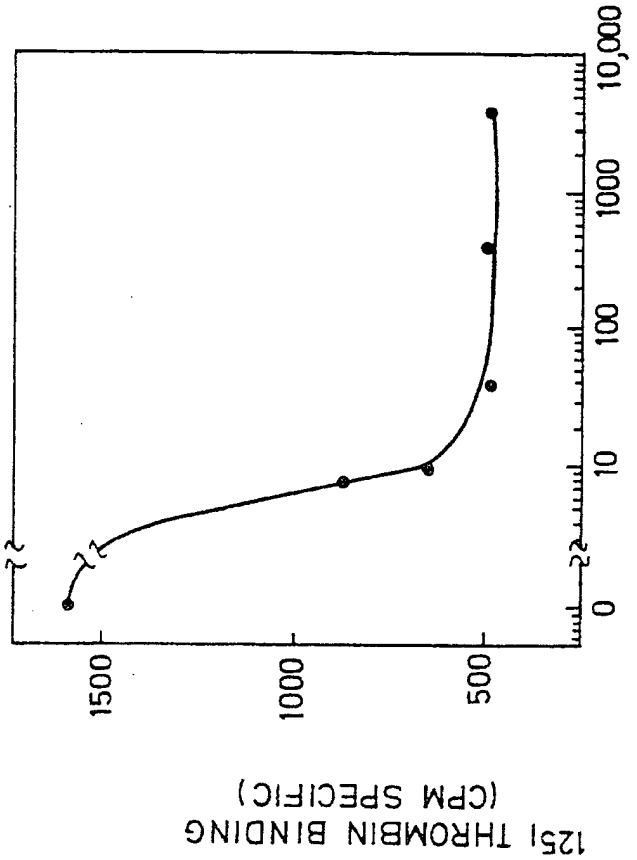

125I THROMBIN BINDING
(CPM SPECIFIC)
1500
1000
500
0
10
100
1000
10,000
p 508-530 (nM)

Fig. 3

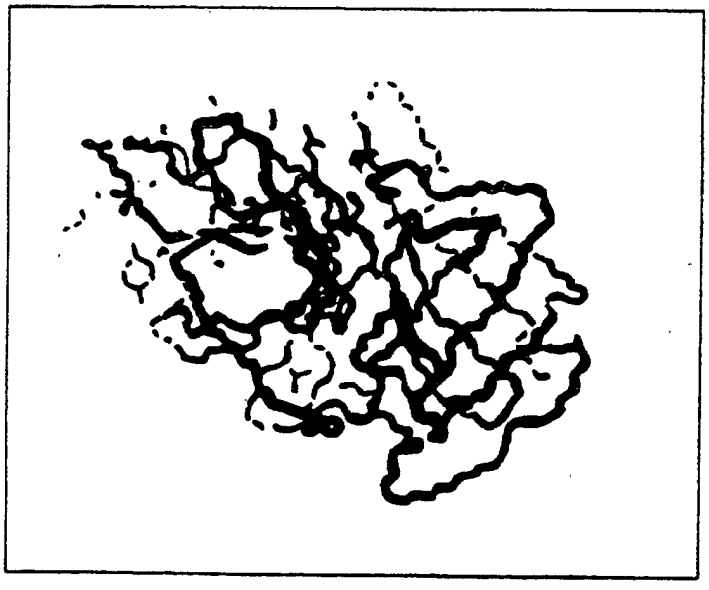


Fig. 2C

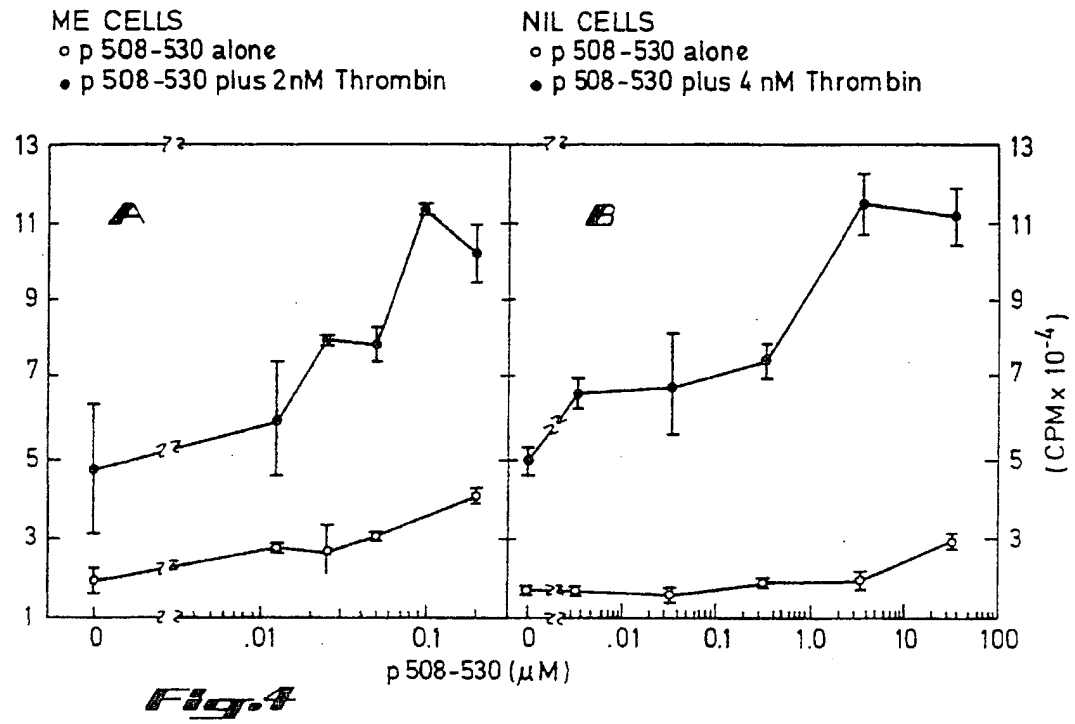

ME CELLS
o p 508-530 alone
• p 508-530 plus 2nM Thrombin
NIL CELLS
o p 508-530 alone
• p 508-530 plus 4 nM Thrombin
A
B
13
11
9
7
5
3
1
0
.01
0.1
1.0
10
100
p 508-530 (μM)
(CPM x 10⁻⁴)

Fig. 4

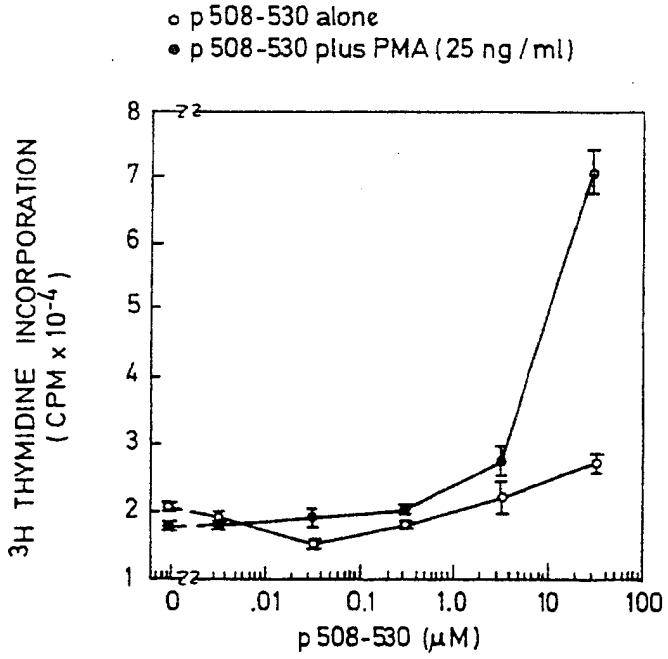
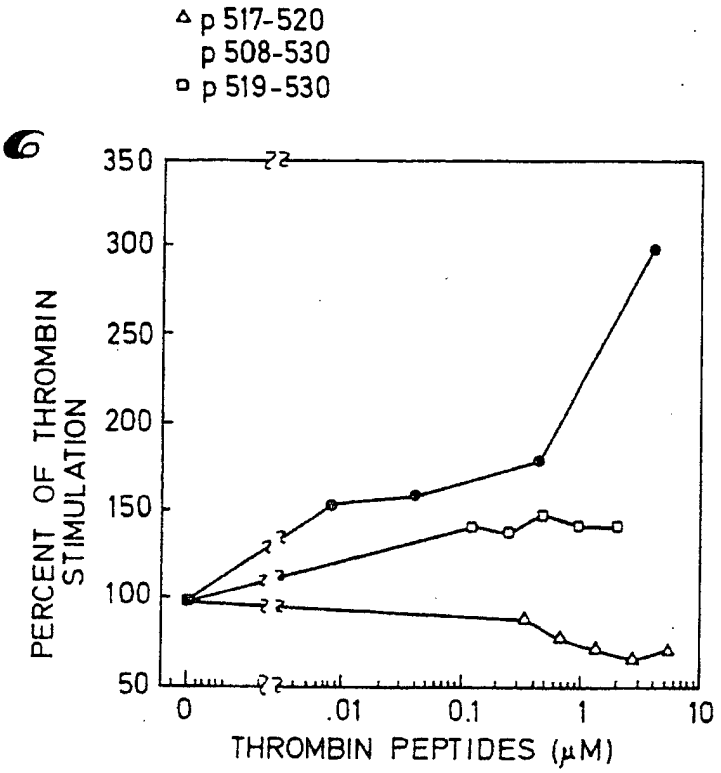

o p 508-530 alone
• p 508-530 plus PMA (25 ng / ml)
³H THYMIDINE INCORPORATION
(CPM x 10⁻⁴)
8
7
6
5
4
3
2
1
0
.01
0.1
1.0
10
100
p 508-530 (μM)

Fig. 5

Fig. 6


△ p 517-520
p 508-530
□ p 519-530
PERCENT OF THROMBIN STIMULATION
350
300
250
200
150
100
50
0
.01
0.1
1
10
THROMBIN PEPTIDES (μM)

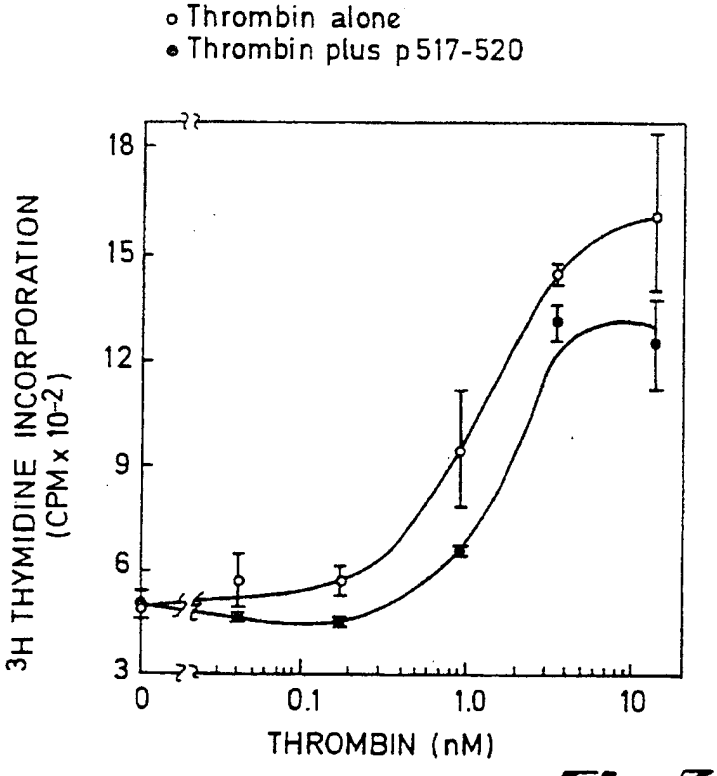

○ Thrombin alone
● Thrombin plus p 517-520
^{3}H THYMIDINE INCORPORATION (CPM x 10^{-2})
18
15
12
9
6
3
0
0.1
1.0
10
THROMBIN (nM)

Fig. 7

THROMBIN DERIVED POLYPEPTIDES; COMPOSITIONS AND METHODS FOR USE

The government owns rights in the present invention pursuant to NIH Research Grant CA00805 and AM25807. FUNDING: Development of the present invention was aided in part by finding from the Department of Health and Human Services, grant nos. DHHS 5R01, AM 25807, and CA 00805. Accordingly, the U.S. Government has a paid-up license and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided by the terms those grants.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention is directed to chemical compounds and methods useful in the regulation of thrombin receptor mediated cell stimulation. More specifically, the invention is directed to prothrombin-derived peptides and methods which employ such peptides for promoting wound healing and inhibiting scar formation, tissue adhesions, blood coagulation, tumor angiogenesis, tumor metastasis and pulmonary edema.

2. Description of the Related Art

Human alpha-thrombin appears to have growth-promoting activity for a wide variety of cells from various tissues. For example, alpha-thrombin has been shown to initiate proliferation of fibroblastic cells in culture without addition of serum or other purified growth factors, to synergize with epidermal growth factor in certain hamster fibroblasts and human endothelial cells, and to initiate cell division or DNA synthesis in mammalian lens epithelial and spleen cells. Yet, the use of thrombin as a growth factor and its potential importance to wound healing has not been widely acclaimed. In part, this may be due to the complexity of thrombin's involvement with coagulation, platelet activation, and initiation of cell proliferation as well as to the complex regulation of thrombin and thrombin-like molecules by serum protease inhibitors and by cell-released protease nexins. This complexity and high degree of physiologic regulation, however, supports the potential importance of this initiation pathway in wound healing.

Thrombin may also play a role in metastasis and angiogenesis of tumors. Generally, for a tumor to grow larger than a few millimeters in diameter, vascular endothelium must proliferate and form vesicle walls to provide circulation and nutrients to the cells inside of the tumor mass. Thrombin likely potentiates this process by virtue of its ability to induce proliferation of endothelial cells. In addition, thrombin has been shown to disrupt the normal intercellular endothelial cell contacts important in preventing cells and plasma factors from escaping or entering the microvasculature. The present hypothesis that thrombin may increase metastasis by disrupting these contacts is supported by studies demonstrating a correlation between decreased levels of anti-thrombin III (which removes thrombin and other proteases from plasma) and increased tumor metastasis.

Various studies have led the present inventors to conclude that high-affinity cell surface thrombin receptors (See Carney and Cunningham, *Cell* 15:1341, 1978) may be involved in tumor metastasis and anglogenesis. For example, studies have indicated that thrombin receptors can serve as binding sites for tissue plasminogen activator, a molecule secreted from metastatic tumor cells. Moreover, other studies demonstrate the involvement of tissue plasminogen activator in metastasis and anglogenesis. Thus, many of the effects of plasminogen activator may be mediated through its interaction with the cell surface thrombin receptor. It is therefore proposed that stimulation of the thrombin receptor serves to promote tumor metastases, while inhibition of the receptor will decrease metastatic activity.

Thrombin has also been shown to cause changes in the structure and function of cells which make up the endothelial vasculature. These studies suggest that thrombin may play a central role in the development of pulmonary edema as well as edema of other tissues. For example, thrombin has been shown to increase permeability of endothelial cell monolayers to macromolecules, to increase arterial pressure and pulmonary vascular resistance, to induce smooth muscle contraction, and to increase transcapillary fluid filtration. All of these effects may be mediated by thrombin's interaction with cell surface thrombin receptors.

A number of recent studies have attempted to elucidate the mechanisms for thrombin-mediated cell stimulation. These studies have indicated to the present inventors that initiation of cell proliferation by thrombin requires two signals. The first signal appears to be dependent upon binding of the thrombin molecule to the high affinity cell surface thrombin receptor, while the second signal results from the enzymic activity of the thrombin molecule. Thus, unlike alpha-thrombin, neither DIP-alpha-thrombin (a proteolytically inactive thrombin derivative that retains receptor-binding activity) nor gamma-thrombin (an esterolytically active, but non-binding thrombin derivative) can initiate DNA synthesis or cell division. However, simultaneous addition of these two non-mitogenic thrombin derivatives initiates a level of DNA synthesis and cell division comparable to that initiated by alpha-thrombin.

These same thrombin derivatives have been used to distinguish intracellular events stimulated by high-affinity thrombin receptor occupancy from those resulting from proteolytic cleavage. Alpha-thrombin and gamma-thrombin both stimulate Na^+/K^+ ATPase activity, phosphoinositol turnover, and Ca^{2+} metabolism, whereas DIP-alpha-thrombin does not. Thus, these events are attributable to thrombin's enzymic activity, not to receptor occupancy. Furthermore, these signals (the release of diacylglycerol and inositol triphosphate to cause Ca^{2+} mobilization) may in turn activate protein kinase C. Accordingly, it has been shown that phorbol myristate acetate (PMA), which activates protein kinase C, can substitute for enzymically active gamma-thrombin and initiate cell division in the presence of receptor saturating levels of DIP-alpha-thrombin or monoclonal antibody to the thrombin receptor. Thus, the requirements for enzymically active thrombin may indirectly relate to its activation of protein kinase C.

The precise signals generated by high-affinity interaction of thrombin with its receptor have been more difficult to define. However, it has recently been shown that DIP-alpha-thrombin stimulates a transient increase in intracellular cAMP. In contrast to ion fluxes and phosphoinositide turnover, cAMP levels are maximally stimulated by DIP-alpha-thrombin but are not stimulated by gamma-thrombin. Attempts to replace DIP-alpha-thrombin with cAMP analogs, however, have been unsuccessful. Therefore, it is possible that throm-

bin receptor occupancy produces a number of signals in addition to changes in cAMP levels.

One problem associated with the clinical application of thrombin directly to achieve such benefits is its susceptability to protease inhibitors by serum anti-thrombins. Such problems have heretofore prevented the use of thrombin in the clinic and has led the present inventors to identify smaller thrombin-active and thrombin antagonistic polypeptides which are not sensitive to the inhibitory effects of thrombin inhibitors.

The present invention provides for a number of smaller polypeptides which have been tailored to interact with the thrombin receptor to selectively stimulate or inhibit thrombin receptor occupancy related signals. It is believed that these polypeptides will prove to be useful in a wide variety of clinical settings where successful recovery may be influenced by thrombin receptor-mediated events.

SUMMARY OF THE INVENTION

The present invention provides a number of thrombin derivatives and methods useful for stimulating cell proliferation and promoting wound healing as well as methods useful in inhibiting wound healing, scar tissue formation, formation of tissue adhesions, and tumor metastasis and anglogenesis. The invention is based on the discovery that one may formulate polypeptide thrombin derivatives, or their physiologically functional equivalents, which selectively inhibit the interaction of thrombin with its high-affinity receptor or which mimic the stimulatory effects of thrombin.

Accordingly, the present invention, in its most general and overall scope, relates to synthetic or naturally derived polypeptide agonists and antagonists of thrombin receptor mediated events. Both of these classes of agents possess a thrombin receptor binding domain which includes a segment of the polypeptide that is capable of selectively binding to the high-affinity thrombin receptor. This segment of the polypeptide includes a sequence of amino acids homologous to a tripeptide cell binding domain of fibronectin.

In addition to the thrombin receptor binding domain, the stimulatory (agonistic) polypeptides possess a sequence of amino acids having sequences derived from the N-terminal amino acids of a dodecapeptide previously shown to be highly conserved among serine proteases. However, the inhibitory polypeptides do not include these serine esterase-conserved sequences.

The present invention is disclosed in terms of a showing that in the presence of a non-mitogenic (i.e., non-stimulatory) concentration of alpha-thrombin, gamma-thrombin, or PMA, the interaction between stimulatory polypeptides and cell surface thrombin receptors provides the cell with a signal to proliferate. However, no proliferative signal results when cell surface thrombin receptors interact with the inhibitory polypeptides. Instead, the cells become more refractory to subsequent treatment with the stimulatory polypeptides. This result is believed to occur because the inhibitory polypeptides, which are themselves incapable of generating a proliferative signal, block binding of the stimulatory polypeptides.

As indicated above, practice of the cell-stimulatory methods of the present invention requires the presence of a secondary signal, for example, in the form of non-mitogenic concentrations of alpha-thrombin, gamma-thrombin, or PMA in order to supply the cells with the low-affinity proteolytic cleavage signal. Accordingly, the invention provides for pharmaceutical compositions and methods to which these compounds have been added. However, those of skill in the art will recognize that when the invention is practiced in vivo, native alpha-thrombin endogenous to the host will typically be adequate to provide this secondary signal.

Because thrombin is involved in a number of bioregulatory effects, the present invention, which allows one to selectively promote and inhibit these effects, has a number of clinical applications. For example, the invention provides a number of polypeptides useful in promoting wound healing. For such applications, the invention provides a polypeptide derivative of thrombin (or a functional equivalent of such a derivative) which has a thrombin receptor binding domain as well as a domain with a serine esterase conserved sequence of at least 12 amino acids. The invention also provides a polypeptide compound of at least 23 L-amino acids which has both a thrombin receptor binding domain and a domain with a serine esterase conserved amino acid sequence.

In one embodiment, the invention provides for several polypeptides containing specific amino acid sequences, such as a polypeptide compound in which the thrombin receptor binding domain includes the L-amino acid sequence Arg-Gly-Asp-Ala together with the serine esterase conserved amino acid sequence, Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val. In a preferred embodiment, the polypeptide compound includes the L-amino acid sequence Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

The invention also provides for a pharmaceutical composition for promoting wound healing which includes of a therapeutically effective concentration of any of the compounds described above combined with a pharmaceutically acceptable excipient. Typically, such compositions include, for example, sufficient concentrations of the polypeptides to effect a stimulatory action on the thrombin receptor as demonstrated herein. Thus, such compositions should typically include sufficient concentrations to obtain levels of the polypeptides in the wound area which are shown in vitro to stimulate the receptor. When endogenous levels of a secondary signal are believed to be inadequate, compositions may be employed which further include the addition of a therapeutically effective concentration of alpha-thrombin or gamma-thrombin.

As used herein, a therapeutically effective concentration is defined as a concentration of the particular agent which provides a satisfactory increase in the rate of wound healing. Again, such concentrations are believed to correspond to levels sufficient to ellicit a stimulation of the thrombin receptor in vitro. However, it is believed that the compositions will prove most effective when the stimulatory (agonistic) polypeptides are present at a concentration of from 0.1 uM to 10 uM.

Furthermore, where alpha-thrombin or gamma-thrombin are also employed, concentrations of from 0.1 uM to 10 uM are considered effective. However, empirical methods as are known in the art may be employed for determining more precisely the proper therapeutic dose for a given composition administered in a particular manner.

In addition, methods are provided which employ thrombin agonists to promote wound healing. One such method includes applying to the wound a therapeutically effective amount of a polypeptide derivative of

thrombin, or a physiologically functional equivalent thereof, which has both a thrombin receptor-binding domain and a domain having a serine esterase conserved amino acid sequence. In general, thrombin is applied in amount sufficient to achieve fibroblast stimulation and thereby stimulate tissue regeneration. In that such methods typically involve topical application to a wound, possible sytstemic toxicity is not believed to be a problem. Therefore, virtually any concentration may be employed. However, in a preferred embodiment, the wound is treated to achieve a range of approximately 1 ng/cm^2–10 ug/cm^2 of wound surface.

The invention further provides a method for promoting wound healing in which a therapeutically effective amount of alpha-thrombin (1 ng/cm^2–10 ug/cm^2 of wound surface) or gamma-thrombin (1 ng/cm^2–10 ug/cm^2 of wound surface) is applied to the wound in conjunction with the foregoing thrombin derivatives. Of course, the specific polypeptides and pharmaceutical compositions provided by the invention may also be used in promoting wound healing. It is believed that these methods will be especially beneficial to patients involved in severe accidents (particularly burn patients), to those subjected to surgical procedures and to those with poor wound healing responses, such as aged and diabetic individuals.

Additional methods are provided for using the thrombin receptor inhibitory polypeptides. For example, the invention provides methods whereby scar tissue formation can be inhibited by administering to the wound or scar tissue, a therapeutically effective amount of a polypeptide derivative of thrombin, or a physiologically functional equivalent thereof, which has a thrombin receptor binding domain but does not have a serine esterase conserved sequence. Typically, such concentrations are adequate when sufficient to inhibit thrombin receptor mediated events. In a preferred embodiment, amounts ranging from 1 ng/cm^2–10 ug/cm^2 of wound surface are considered appropriate.

In a preferred embodiment, the polypeptide derivative of thrombin has the L-amino acid sequence Arg-Gly-Asp-Ala.

In general, these methods may be used in any situation where scar formation is undesirable, such as on burn patients or those subjected to opthalmic surgery. Moreover, the methods may also be of use in preventing keloidal scar formation. It is anticipated that spraying the wound with an aerosol spray will be a particularly sterile and efficacious manner of administering the polypeptide compound to the wounds of burn patients.

The inhibitory polypeptides should also prove useful in inhibiting the formation of tissue adhesions, defined as abnormal unions between body organs by formation of fibrous tissue. It is known that fibroblast proliferation is required for formation of such adhesions. Since alpha-thrombin is known to induce fibroblast proliferation, it follows that inhibition of thrombin-mediated mitogenesis by the peptides of the present invention could reduce adhesion formation. It is believed that administration of such inhibitory polypeptides to the surface of the affected organs will prove to be especially useful following certain surgical procedures, such as thoracic surgery, where gut adhesions often lead to postoperative complications.

It is further proposed that the inhibitory peptides will prove useful in the treatment of mammals with tumors to thereby inhibit tumor metastasis or angiogenesis. This view is supported by studies demonstrating that alpha-thrombin is able to disrupt normal inter-endothelial cell contacts important in preventing metastasis, as well as studies demonstrating that alpha-thrombin can induce the proliferation of endothelial cells required for angiogenesis. Accordingly, the invention provides a method whereby mammals with such tumors receive a therapeutically effective amount of a polypeptide derivative of thrombin, or a functional equivalent thereof, which has a thrombin receptor binding domain but does not have a serine esterase conserved sequence. While exact doses would need to be determined by empiracal methods known those skilled in the art, it is estimated that an amount sufficient to achieve a concentration of from 0.1 uM to 10 uM at the site to be treated is needed. Use of a polypeptide wherein the thrombin binding domain has an L-amino acid sequence Arg-Gly-Asp-Ala is specifically provided. It is contemplated that the polypeptides will be most efficacious in this regard when administered intravenously. However, other methods of administration will also likely prove to be effective.

In a most general embodiment, the invention provides for the use of inhibitory polypeptides to inhibit cell proliferation. This method encompasses, but is not limited to, situations in which one desires to inhibit cell proliferation in vitro. Of course, the inhibitory polypeptide, having a thrombin binding domain with the specific sequence Arg-Gly-Asp-Ala, may also be used as a general inhibitor of cell proliferation.

In another general embodiment, the invention comprises methods wherein the stimulatory polypeptides are used to potentiate cell growth. A polypeptide including the sequence Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-phe-Val is specifically provided. This method encompasses, but is not limited to, situations wherein one wishes to potentiate cell growth in vitro. Such cell-stimulatory uses may be potentiated by further providing an effective amount of alpha-thrombin (0.1 ug/ml–10 ug/ml), gamma-thrombin (0.1 ug/ml–10 ug/ml) or phorbol myristate acetate (10 ng/ml–100 ng/ml) in conjunction with the stimulatory polypeptide.

GLOSSARY

For purposes of the present invention, a thrombin derivative is defined as any molecule with an amino acid sequence derived at least in part from that of thrombin, whether synthesized in vivo or in vitro. Accordingly, a thrombin derivative, as referred to herein, designates a polypeptide molecule which comprises fewer amino acids than thrombin.

A physiologically functional equivalent of a thrombin derivative encompasses molecules which differ from thrombin derivatives in particulars which do not affect the function of the thrombin receptor binding domain or the serine esterase conserved amino acid sequence. Such particulars may include, but are not limited to, conservative amino acid substitutions and modifications, for example, amidation of the carboxyl terminus, acetylation of the amino terminus, conjugation of the polypeptide to a physiologically inert carrier molecule, or sequence alterations in accordance with the serine esterase conserved sequences.

A thrombin receptor binding domain is defined as a polypeptide sequence which directly binds to the thrombin receptor and/or competitively inhibits bind-

ing between high-affinity thrombin receptors and alpha-thrombin.

A domain having a serine esterase conserved sequence comprises a polypeptide sequence containing at least 4–12 of the N-terminal amino acids of the dodecapeptide previously shown to be highly conserved among serine proteases (Asp-X_1-Cys-X_2-Gly-Asp-Ser-Gly-Gly-Pro-X_3-Val); wherein X_1 is either Ala or Ser; X_2 is either Glu or Gln; and X_3 is either Phe, Met, Leu, His, or Val).

A stimulatory polypeptide is defined as a polypeptide derivative of thrombin, or a physiologically functional equivalent thereof, having the ability to both bind to and stimulate the thrombin receptor. Therefore, the stimulatory peptides will include both a thrombin receptor binding domain and a domain with a serine esterase conserved amino acid sequence.

An inhibitory polypeptide is defined as a polypeptide derivative of thrombin, or a physiologically functional equivalent thereof, having a thrombin receptor binding domain but without a serine esterase conserved amino acid sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1D. Computer assisted analysis of the hydropathy, solubility, and predicted secondary structure for residues 489 to 548 of human prothrombin. FIG. 1A, hydropathy profile; FIG. 1B, solubility profile; FIG. 1C, predicted tendency for flexible turn; FIG. 1D, predicted tendency for alpha-helix and beta-sheet structure.

FIGD. 2A-2C. Three-dimensional representations of X-ray crystallographic data of trypsin with the following PROTEUS computer-assisted substitutions of thrombin-specific residues: Gly_{187} Lys; Lys_{188} Arg; Ser_{190} Ala; Gln_{192} Glu; and Val_{199} Phe is shown in FIG. 2A. FIG. 2B and 2C show only the three active site residues (His_{57}, Asp_{102}, Ser_{195}) and residues 183 to 200 of trypsin that are located in the homologous region as thrombin's residues 510 to 530. These peptides are oriented in the same position as in the rotated model in FIG. 2A.

FIG. 3. Inhibition of [^{125}I]-alpha-thrombin binding to mouse embryo (ME) cells by synthetic peptide p508–530. Specific binding of 0.3 nM [^{125}I]-alpha-thrombin to ME cells in the presence of the indicated concentration of peptide was measured as described in the description of the preferred embodiments.

FIG. 4 Effect of p508–530 on [^{3}H]-thymidine incorporation alone or in combination with low concentrations of alpha-thrombin. Quiescent serum-free cultures of ME (FIG. 4A) or NIL (a hamster fibroblast cell line; FIG. 4B) were treated with the indicted concentrations of p508–530 alone (O) or in combination with concentrations of alpha-thrombin which gave approximately one third of the maximal response; 2 nM for ME cells (FIG. 4A) and 4 nM for NIL cells (FIG. 4B). [^{3}H]-thymidine incorporation was determined after 24 hours as described in the description of the preferred embodiments.

FIG. 5 Effect of p508–530 on [^{3}H]-thymidine incorporation in combination with PMA. Quiescent cultures of NIL cells were incubated with p508–530 alone (O) or in combination with 25 ng/ml PMA. [^{3}H]-thymidine incorporation was determined as described in the description of the preferred embodiments.

FIG. 6. Comparison between effects of peptides on thrombin-stimulated thymidine incorporation. Quiescent cultures of NIL cells were incubated with increasing concentrations of p508–530, p519–530, or p517–520 in the presence of 1 nM alpha-thrombin (a marginally mitogenic concentration). Data are expressed for each concentration as a percentage of the effect of alpha-thrombin alone.

FIG. 7. Effect of p517–520 on thrombin stimulation of [^{3}H]-thymidine incorporation. Quiescent cultures of ME cells were incubated with increasing concentrations of alpha-thrombin alone, or in combination with 625 nM p517–520. [^{3}H]-thymidine incorporation was determined as described in the description of the preferred embodiments.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Thrombin, a molecule once considered important only in the context of blood coagulation, is now shown to mediate a number of potent biological effects not directly related to coagulation. Many of these effects are due, at least in part, to signals generated by the interaction between thrombin or thrombin-like molecules and the high-affinity thrombin receptors present on the surface of many cells.

Studies performed in connection with the present invention suggested that selective regulation of thrombin-mediated events might be achieved through the formulation and synthesis of polypeptides specifically designed to either mimic or inhibit such events. Development of small protease inhibitor resistant polypeptides capable of performing these functions was particularly desirable in view of the susceptibility of thrombin to proteolytic enzyme inhibitors, such as anti-thrombin.

A number of peptides based on the sequence of human prothrombin were synthesized and tested for their ability to bind to the receptor and to generate proliferative signals. The choice of peptides focused on the amino acid sequence of the region of thrombin around its active site serine. This region contains a domain (represented by residues 517–520 of human prothrombin) with a sequence homologous to the tripeptide cell binding domain of fibronectin, [Arg-Gly-Asp]. This tripeptide sequence is common to a number of proteins that may interact with cells (reviewed by Rouslahti and Peirschbacher, *Cell,* 44:517–518 (1985)). Moreover, it has been shown that a peptide representing 517–520 of human prothrombin (p517–520) and peptides representing 516–522 and 510–526 of human prothrombin (p516–522 and p510–526, respectively) are able to promote fibroblast attachment comparable to that induced by fibronectin-specific peptides.

The selected region also possesses a domain (represented by residues 519–530 of human prothrombin) with a high degree of homology to a number of serine esterases.

The present inventors have discovered that a synthetic peptide containing both fibronectin- and serine protease-homologous domains (residues 508 to 530 of human prothrombin) binds to thrombin receptors with high-affinity and substitutes for DIP-alpha-thrombin as an initiator of receptor occupancy-related mitogenic signals. In contrast, a synthetic peptide containing only the fibronectin-homologous domain (p517–520) binds to the thrombin receptor without inducing mitogenesis. An intermediate peptide (p519–530) retains the ability to mediate mitogenesis but to a much lesser degree than p508–530.

EXAMPLE 1

Selection of Domains of Human Alpha-Thrombin Involved in Binding of Thrombin to Its High Affinity Receptor

To help select peptide sequences that might be involved in receptor binding, computer analysis was used to predict the overall hydropathy, solubility, and secondary structural features for the **60** amino acid residues around the active site serine of alpha-thrombin based on the sequence of human prothrombin (Degen et al., *Biochem.*, 2:2087–2097 (1983)). As shown in FIGS. **1A** and **1B**, this region appears to be highly hydrophilic and soluble, especially near the region that is homologous to fibronectin's cell attachment domain, residues 517 to 520. Analysis of secondary structural features indicated that the region of thrombin from residues 511 to 526 has a strong tendency for being a flexible turn region with very little tendency towards either alpha-helical or beta-sheet structures (FIGS. **1C** and **1D**). Taken together, the various computer-assisted analyses strongly suggest that this region of thrombin should be externally accessible and, therefore, available for interaction with the thrombin cell surface receptor. Moreover, the region of thrombin homologous to the cell attachment domain of fibronectin is located at or very near the middle of this hydrophilic flexible turn of thrombin.

Using the three dimensional x-ray crystallographic data for trypsin (Marquart et al., *Acta. Crystallogr.*, 39:480 (1983)), and making appropriate amino acid substitutions to reflect the thrombin sequence around the active-site serine portion of trypsin, computer graphic analysis predicted that residues 510 to 530 of thrombin are located along the edge of the pocket that leads to the active site cleft (FIG. **2**). In agreement with the predictions of secondary structure discussed above, amino acid residues **517** to **520** of thrombin are located at the outer most corner of this region of the proposed trypsin/thrombin structure. Thus, it appeared reasonable that this region of thrombin could be involved in binding to its receptor.

EXAMPLE 2

Synthesis of Peptides

Peptides were synthesized by the solid-phase method (Erickson and Merrifield, *The Proteins*, 2:255–257, (1976)) using automatic instrumentation (Applied Biosystems Peptide Synthesizer Model 430A) and purified by HPLC (Beckman) on a C-18 column eluted with a linear acetonitrile gradient containing 0.5% (v/v) TFA (trifluoroacetic acid).

EXAMPLE 3

Demonstration that the Thrombin Derivatives Selectively Bind to the High-Affinity Thrombin Receptor

This example demonstrates that the peptides of the present invention are able to selectively bind to the high-affinity thrombin receptors present on the surfaces of many cell types. In the present embodiment, this activity was demonstrated by showing that the peptides of the present invention competitively inhibited binding of [^{125}I]-alpha-thrombin to thrombin receptors present on two strains of cultured fibroblasts. Accordingly, the specific techniques described below represent the best mode for demonstrating this activity known to the inventors at the present time.

a. Culture of Fibroblasts Having High-Affinity Thrombin Receptors

As stated above, fibroblasts derived from two sources were used to demonstrate binding of the peptides of the present invention to high-affinity thrombin receptors. These cell lines were prepared as follows:

Primary cultures of fibroblasts were prepared from 9- to 13-day old embryos of NIH-swiss outbred mice as described by Carney and Cunningham, *Cell*, 15:1341–1349, (1978). NIL cells, an established strain of hamster fibroblasts, were maintained as stock cultures and subcultured every four days. All cells were grown in Dulbecco-Vogt modified Eagle's (DV) medium supplemented with 10% (v/v) bovine calf serum (CS), in a humidified atmosphere of 5% CO_2 in air at 37° C.

Quiescent cultures were prepared by subculturing stock cells from 100 mm dishes, using 0.05% (w/v) trypsin and 0.02% EDTA (w/v) in phosphate-buffered saline (PBS) and plating them in 24-well culture plates in DV medium supplemented with 10% (v/v) CS at 6×10^4 *cells/cm2*. After allowing the cells to attach overnight, the medium was removed and the cells were rinsed with DV medium containing no serum. The cells were incubated in this serum-free medium for 48 hours before the indicated experiments. This procedure has been shown to provide nonproliferating populations of mouse and NIL cells that are 90–95% arrested at the G_1/G_0 cell cycle interface.

b. Assay for Measurement of Specific Binding of Thrombin and Thrombin Derivatives to the Cell Surface Thrombin Receptor

As stated above, in the present embodiment, thrombin receptor specific binding activity of the thrombin derivatives was measured as a function of their ability to competitively inhibit binding between native [^{125}I]-thrombin and the thrombin receptor. Specific techniques whereby the competitive binding studies were performed are set out below.

Human alpha-thrombin was iodinated in the presence of benzamidine (an active-site competitive inhibitor), lactoperoxidase, and Na[^{125}I]. After gel filtration and dialysis, the [^{125}I]-alpha-thrombin had a specific activity of 1 to 3×10^{-7} CPM/ug and co-migrated with unlabeled alpha-thrombin as a single band on sodium dodecyl sulfate (SDS) polyacrylamide gels. These iodinated preparations retained approximately 80% of their fibrinogen clotting activity.

The ability of the synthetic peptides to compete for specific [^{125}I]-alpha-thrombin binding to fibroblasts was measured on nonproliferating, mitogenically responsive cultures in 24 well plates (Falcon) at a cell density of approximately 5×10^4 cells/cm^2 as previously described (Carney and Cunningham, *Cell*, 15:1341–1349 (1978)). The medium on the cells was changed to binding medium (serum-free DV medium containing 0.5% (w/v) bovine serum albumin buffered with 15 mM HEPES at pH 7.0). The cells were equilibrated at 23° C. for 30 minutes, and the medium was changed to binding medium containing [^{125}I]-alpha-thrombin (10 ng/ml) with the indicated concentrations of the peptides. After 2 hours at 23° C., the assay was terminated by quickly rinsing the cells four times with ice-cold PBS. The cells were dissolved in 1 ml of 0.5 N NaOH and the total radioactivity was measured using a Beckman gamma counter. Nonspecific binding was measured as the radioactivity bound to cultures after incubation in binding

medium containing a 100-fold excess of unlabeled alpha-thrombin. Specific binding was calculated by subtracting nonspecific binding from total radioactivity bound to cultures.

c. Thrombin Binding Activity of Selected Thrombin Derivatives

In order to demonstrate the thrombin receptor binding activity of the polypeptides of the present invention, the peptides synthesized as described in Example 1 were tested for thrombin receptor activity using the assay system described immediately above.

More specifically, in order to demonstrate that p508–530 bound to thrombin receptors, confluent cultures of ME cells were incubated with 0.3 nM [^{125}I]-alpha-thrombin and concentrations of p508–530 ranging from 8 to 4000 nM for 90 minutes at 23° C. As shown in FIG. 3, p508–530 competed for 30% to 70% of the specific binding of [^{125}I]-alpha-thrombin to ME cells. Scatchard analysis of the direct binding of [^{125}I]-labeled p508–530 indicated a K_D of approximately 6×10^{-8} M (data not shown). In addition, the specific binding of [^{125}I]-p508–530 to ME cells could be displaced by both excess p508–530 or excess human alpha-thrombin. Thus, it appears that the competition of p508–530 for [^{125}I]-alpha-thrombin binding represents the binding of p508–530 to the same sites as alpha-thrombin, but with an affinity approximately one order of magnitude lower.

Furthermore, in order to show that the binding and mitogenic activity of p508–530 was specific, two synthetic peptides with physical properties similar to p508–530 but no sequence homology to human alpha-thrombin were tested for their binding properties. Both of these peptides [one with 12 amino acids (33% hydrophobic residues and a net charge of −3) and a second with 18 amino acids (39% hydrophobic residues and a net charge of 0)] inhibited binding of [^{125}I]-alpha-thrombin less than 5% at concentrations up to 5 uM.

To further identify regions of thrombin involved in high-affinity binding and generation of mitogenic signals, two peptides representing specific domains within p508–530 were tested. The first peptide represented residues 519 to 530 of the B-chain region of human prothrombin, a region of thrombin that is highly conserved among serine proteases. The second peptide represented residues 517 to 520 of prothrombin, a region of thrombin homologous to the fibronectin cell binding domain.

Both of these peptides were able to compete for 30% to 50% of the binding of [^{125}I]-alpha-thrombin to ME cells, but both required higher concentrations than was required with the initial peptide p508–530 (Table 1). For example, 30% inhibition of [^{125}I]-alpha-thrombin binding required 33- to 50-fold higher concentrations of p519–530 and p517–520 than p508–530, respectively. Thus, both of these peptides appear to interact with thrombin receptors, but at a lower affinity than p508–530. Because p517–520 is homologous to the fibronectin cell binding domain, a peptide having the sequence Arg-Gly-Ala-Ser (the sequence of the fibronectin specific peptide) was also tested for its ability to compete for [^{125}I]-alpha-thrombin binding. At a concentration of 1.3 uM, this peptide did not compete with [^{125}I]-alpha-thrombin for binding. Thus, the receptor for alpha-thrombin is not the same membrane protein that specifically interacts with fibronectin and causes the apparent growth promoting action of fibronectin. In addition, these results demonstrate the requirement for alanine within the thrombin receptor binding domain, since substitution of alanine with serine eliminated the ability of the synthetic peptide to compete for alpha-thrombin binding.

TABLE 1

Comparison of Peptide Competition for [^{125}I]-Alpha-Thrombin Binding to ME Cells.

Peptide	Amino Acid Sequence	Concentration Required for 30% Inhibition	Maximal % Inhibition (and Conc.)
p508-530	AGYKPDEG— —KRGDACE— —GDSGGPFV	6 nM	78% (40 nM)
p519-530	DACEGD— —SGGPFV	200 nM	51% (800 nM)
p517-520	RGDA	300 nM	50% (2.7 uM)

Various concentrations of peptides and [^{125}I]-alpha-thrombin (1 nM) were incubated with quiescent ME cells for 90 minutes at 23° C. Specific binding of [^{125}I]-alpha-thrombin was defined as described in Example 3.

EXAMPLE 4

Stimulation of DNA Synthesis by Selected Thrombin Derivatives

This example demonstrates that binding between stimulatory (agonistic) polypeptides and thrombin receptors generates a receptor occupancy signal which induces DNA synthesis and cell division. In the present embodiment, DNA synthesis and cell proliferation was measured as a function of [^{3}H] thymidine uptake by cultured fibroblasts exposed to selected polypeptides in the presence of non-mitogenic concentrations of alpha-thrombin or PMA. Although the in vitro techniques described below represent the best mode for demonstrating the stimulatory activity of the selected polypeptides, those skilled in the art will appreciate that the principles demonstrated in the in vitro system described immediately below are also applicable in vivo.

a. Techniques for Measuring DNA Synthesis

The effects of the synthetic peptides on DNA synthesis were determined by measuring the incorporation of methyl-[^{3}H]-thymidine (TdR, ICN Pharmaceuticals, Irvine, Ca.) during a 2 hour incubation generally from 22 hours after addition of peptides and/or thrombin (Stiernberg et al., *J. Cell Physiol.*, 120:209–285 (1984)). After incubation, the cells were extracted and rinsed with ice-cold 10% (w/v) trichloroacetic acid (TCA). The acid precipitable material was dissolved overnight in 0.5 ml 0.5 N KOH at 23° C. HCl (1 N), 0.25 ml, was added and the solution was counted in 10 ml of RediSolv-HPb (Beckman Instruments, Houston, Tex.) scintillation fluid.

b. Mitogenic Activity of Selected Thrombin Derivatives

Each of the thrombin derivatives synthesized was tested for mitogenic activity as were the two non-thrombin peptides described in Example 3(c). The results of these experiments are described below.

The present inventors first tested the ability of p508–530 to stimulate DNA synthesis in non-proliferating cultures of ME or NIL cells. As shown in FIG. 4, p508–530, by itself, was not sufficient to stimulate [^{3}H]-thymidine incorporation into DNA. However, in combination with 2 nM alpha-thrombin, 0.1 uM p508–530 stimulated a 6- or greater than 2-fold increase in incor-

poration of [^{3}H]-thymidine into DNA in ME cells when compared to parallel cultures left untreated or treated with alpha-thrombin alone, respectively (FIG. 4A). A similar mitogenic stimulation was also observed in NIL hamster cells, although it required a slightly higher concentration of thrombin and peptides (FIG. 4B). The responses in both cell types were equivalent to the mitogenic response stimulated by a maximally effective concentration of alpha-thrombin (10 nM). It is noteworthy that for ME cells, stimulation by p508–530 was observed between 12.5 nM and 100 nM (FIG. 4A), concentrations that correspond closely with those required to inhibit [^{125}I]-alpha-thrombin binding to ME cells (FIG. 3). With NIL cells, a similar correlation was observed between the mitogenic concentrations of p508–530 and the concentrations required to inhibit thrombin binding, although at higher levels than required with ME cells.

Although these results suggest that p508–530 generates mitogenic signals through its interaction with high-affinity thrombin receptors, it was possible that the peptide merely increased the effective concentration of alpha-thrombin. Recently, phorbol myristate acetate (PMA) has been shown to mimic the effects of gamma-thrombin and stimulate DNA synthesis and cell proliferation in combination with DIP-alpha-thrombin or with monoclonal antibodies to the thrombin receptor. It was predicted, therefore, that if p508–530 was generating a receptor occupancy-related signal, its addition to cells in combination with PMA should stimulate mitogenesis. As shown in FIG. 5, in the presence of 25 ng/ml PMA (which is a non-mitogenic amount), p508–530 stimulated a 3.5-fold increase in DNA synthesis over controls. This stimulation occurred at approximately the same concentration of peptide as that required to stimulate DNA synthesis in the presence of low concentrations of alpha-thrombin. Since active thrombin was not present in these experiments, it would appear that p508–530 itself generates a mitogenic signal that mimics the effect of DIP- or alpha-thrombin binding to high-affinity thrombin receptors.

In order to ensure that the stimulation of DNA synthesis by p508–530 was mediated by virtue of its ability to interact with the high-affinity thrombin receptor, the synthetic, non-thrombin, non-receptor binding polypeptides described in Example 3(c) were tested for mitogenic activity. Neither of these peptides generated a mitogenic response in the presence of 1 nM alpha-thrombin. Thus, neither the binding activity nor the mitogenic activity of p508–530 is due to non-specific interaction of the polypeptide with the cells.

The inventors then tested the mitogenic activity of the smaller thrombin derivatives, p519–530 and p517–520. As indicated in Example 3(c) above, both of these peptides bind to the high-affinity thrombin receptor. In these experiments, increasing concentrations of p519–530 and p517–520 were added to quiescent NIL cells in the presence of 2 and 4 nM alpha-thrombin. As shown in FIG. 6, p519 enhanced DNA synthesis over a range of concentrations while p517–520 did not. In fact, p517–520 actually inhibited DNA synthesis.

EXAMPLE 5

Inhibition of Thrombin-Receptor Mediated Mitogenesis by p517–520

The observation that p517–520 inhibits alpha-thrombin stimulated mitogenesis was somewhat startling in view of previous studies demonstrating that mitogenic and transmembrane signaling effects of thrombin were not inhibited by DIP-alpha-thrombin, a thrombin derivative which competes for active alpha-thrombin binding. Thus, the inventors realized that p517–520, which is able to compete with native alpha-thrombin for binding to high-affinity cell surface thrombin receptors, but is unable to generate the mitogenic receptor occupancy signal, has properties not previously known in the art.

In order to explain the mechanism by which p517–520 was able to inhibit thrombin-mediated mitogenesis, the inventors measured the ability of increasing concentrations of alpha-thrombin to stimulate DNA synthesis in cultures to which a constant concentration (625 nM) of p517–520 had been added (FIG. 7). These experiments showed that p517–520 significantly shifted the dose-response curve of the cells to alpha-thrombin. For example, at two concentrations of alpha-thrombin, 0.8 and 13.0 nM, DNA synthesis was inhibited by approximately 75% and 35%, respectively. Thus, the inhibition of alpha-thrombin stimulation by p517–520 appears to require a 500–1000 fold molar excess of the peptide. This finding is consistent with the observation that p517–520 has a lower competitive binding affinity for thrombin receptors on ME cells than does p508–530.

The identification of p517–520 as the high-affinity binding domain of thrombin has several implications as to the mechanism of thrombin mitogenesis. Previous studies have demonstrated proteolytic cleavage and disappearance of a molecule on the surface of chick embryo cells treated with thrombin. Cross-linking studies with active or inactive thrombin have also identified two differently sized receptor molecules or substrates. The present results show that the high-affinity binding domain of thrombin is very close to the active-site cleft; thus, it should be possible for thrombin to cleave its receptor. Preliminary data from affinity purification of the thrombin receptor supports the hypothesis that the receptor itself is proteolytically cleaved by active thrombin. It is possible that thrombin receptor occupancy may stimulate an alteration in receptor conformation necessary for the cleavage event. The present results suggest that peptides p508–530, p519–530 or alpha-thrombin itself are capable of binding to the thrombin receptor in a manner which induces such confirmational changes. In contrast, p517–520 appears to be capable only of binding to the receptor. Thus, p517–520 selectively inhibits thrombin receptor-mediated events by virtue of its ability to selectively interact with thrombin receptors in a manner which provides the cell with a null signal.

EXAMPLE 6

Use of Stimulatory Peptides to Potentiate Cell Growth In Vitro

A number of experimental and diagnostic procedures require cells grown in vitro. Because the stimulatory peptides enhance proliferation of fibroblastic cells bearing high-affinity thrombin receptors, the incorporation of such stimulatory molecules into the culture medium will provide an effective means of potentiating cell growth. In addition, because thrombin stimulates proliferation of other cells, including endothelial cells, these peptides may be effective in promoting growth of a number of types of cells. Use of the synthetic polypeptides as growth supplements has a number of advantages. It is much less expensive to synthesize the polypeptides than its is to purify naturally occurring throm-

bin. Furthermore, unlike naturally occurring thrombin, the polypeptides are relatively resistant to inhibition by serum protease inhibitors.

Numerous methods for preparing cells for culture are known to those skilled in the art. One such method, described by Carney et al. (*J. Cell. Physiol.*, 95:13–22, 1978, incorporated herein by reference), is believed to be particularly well suited to the practice of this aspect of the invention.

As will be appreciated by those of skill in the art, the stimulatory polypeptides of the present invention may be employed together with any suitable cell culture medium to achieve the advantages of their cell-stimulation effects. For example, the present inventors have found a mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F12 medium to be a particularly appropriate base medium. To practice the invention, one adds 0.1 ug/ml–10 ug/ml of the stimulatory peptide Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val to the culture medium. The cells are then incubated in an appropriate humidified atmosphere, for example, one containing 5% CO_2 in air at 37° C. At regular intervals (3 or 4 days), the spent medium is removed from the cell culture and replaced with fresh medium formulated as described above.

EXAMPLE 7

Treatment Protocols

Due to precautions necessarily attendant to development of every new pharmaceutical, the polypeptides of the present invention have not yet been tested in a clinical setting in human subjects. However, the in vitro activity of these polypeptides in selectively promoting or inhibiting thrombin-mediated mitogenesis is believed to demonstrate the utility of the present invention in this regard. The following prophetic embodiments represent the best mode contemplated by the present inventors of carrying out the practice of the invention in various clinical settings.

a. Wound Healing

It is believed that the stimulatory polypeptides will prove to be useful in numerous clinical situations where it is desirable to potentiate wound healing. In particular, these include treatment of burn patients, those involved in severe accidents, those subjected to a variety of surgical procedures and those with poor wound healing responses, such as the aged and diabetic. Although the best mode of administering the polypeptides will depend on the particular clinical situation, it is believed that its administration in the form of an aerosol spray will prove to be particularly advantageous in a number of such settings. Methods for incorporating therapeutic agents into aerosol sprays are well known in the art. Therefore, it is considered that formulation and use of these stimulatory polypeptides in such aerosol sprays is well within the skill of the art in light of the present disclosure.

The stimulatory polypeptide may also be applied to the wound in the form of a salve or lotion. Alternatively, they may be incorporated into the material used to dress the wound. Techniques for incorporation of therapeutic agents compositions into salves, lotions and wound dressings are also well known in the art and within the skill of the art in light of the present specification.

It is believed that an effective dose of the polypeptide is approximately between 0.5 uM–50 uM. However, exact dosages would, of course, be determined empirically by experimental methods well known to those skilled in the pharmaceutical arts.

b. Use of the Inhibitory Polypeptides

1. Inhibition of Scar Formation and Formation of Tissue Adhesions

It is further believed that the inhibitory polypeptides will prove useful in a number of situations, for example, where inhibition of fibroblast proliferation is desirable. These include prevention of scar formation and tissue adhesions.

One manner in which the invention may be practiced is by incorporating the inhibitory polypeptide Arg-Gly-Asp-Ala into any vehicle suitable for application to a wound, surgical incision or surface of a body organ. These vehicles include aerosol sprays, salves and lotions appropriate for direct application to tissues as well as solutions appropriate for intravenous or subcutaneous injections. Methods for incorporating therapeutic agents into pharmaceutical vehicles such as those described above is believed to be well within the skill of the art, as are methods for applying the resultant compositions.

It is proposed that an effective dose of the polypeptide is 1 ng/cm^2–10 ug/cm^2 if the compound is applied topically. If injected, an effective dose is that dose sufficient to obtain a concentration of the polypeptides of from 0.1 uM to 10 uM, at the site where needed. However, exact doses, of course, should be determined by accepted pharmaceutical methods known to those skilled in the pharmaceutical arts.

2. Tumor Therapy

It is believed that the inhibitory polypeptides will further prove to be useful in the treatment of various tumors, particularly in preventing metastasis and anglogenesis. It is anticipated that the inhibitory polypeptides could best be administered by intravenous administration.

The inhibitory polypeptides could be given daily by continuous infusion or on alternative days, with more traditional chemotherapy being given on the intervening day. While exact doses of the inhibitory peptides would have to be determined empirically by methods known to those skilled in the art, it is estimated that an effective dose would be that amount sufficient to achieve a concentration of 0.1 uM to 10 uM at the site where needed. Of course, as with a new pharmaceutical agent of any type, clinical trials would be needed to establish levels at which unacceptable toxicity would be reached.

The present invention has been disclosed in terms of specific embodiments believed by the inventor to be the best mode for carrying out the invention. However, in light of the disclosure hereby provided, those of skill in the various arts will recognize that modifications can be made without departing from the intended scope of the invention. For example, any of these peptides may be administered by a number of methods known in the art. Furthermore, future studies are expected to result in production of thrombin derivatives with increased stimulatory or inhibitory activity. These and all other modifications and embodiments are intended to be within the scope of the claims.

What is claimed is:

1. A polypeptide consisting essentially of a thrombin receptor binding domain and a serine esterase conserved sequence wherein said polypeptide comprises 23 amino acids.

2. A polypeptide thrombin derivative consisting essentially of: Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

3. A polypeptide consisting of the amino acid sequence Ala-Gly-Tyr-Lys-Pro-Asp-Glu-Gly-Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

4. A composition of matter comprising:

a substantially purified thrombin derivative peptide or physiologically functional equivalent thereof of 23 amino acids in length wherein said peptide includes;

(a) a thrombin receptor binding domain having the sequence Arg-Gly-Asp-Ala; and

(b) a serine esterase conserved sequence;

and wherein the Asp-Ala of the thrombin receptor binding domain comprise the first two amino acids of the serine esterase conserved sequence.

5. The composition of claim 4 wherein the serine esterase conserved sequence is Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

6. The composition of claim 4 wherein the serine esterase conserved sequence comprises AspAla-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-Val.

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